CATIONIC LIPID-MEDIATED ENHANCEMENT OF NUCLEIC ACID IMMUNIZATION OF CATS

FIELD OF THE INVENTION

The present invention relates to a method to introduce a nucleic acid molecule into a felid by administration of a nucleic acid molecule-cationic lipid complex composition. In particular, the present invention relates to the parenteral administration of a nucleic acid molecule-cationic lipid complex to elicit and/or enhance an immune response to the protein encoded by the administered nucleic acid molecule.

BACKGROUND OF THE INVENTION

Introduction of DNA into an animal for the purpose of eliciting an immune response is often referred to as DNA vaccination. DNA vaccination represents a means of expressing an antigen *in vivo* for the generation of humoral and cellular immune responses. DNA vaccines employ genes encoding antigens, rather than using the proteins themselves, to induce immune responses. The DNA, upon administration to the host, is transcribed and translated *in vivo* to produce an antigen. Processing and presentation of the antigen stimulates the animal's immune system to elicit a humoral and/or cellular response to the antigen. This immune response can potentially confer protective immunity to the animal.

DNA vaccines appear to have advantages over protein antigen-based vaccines, standard "killed" pathogen vaccines, live, attenuated vaccines, and recombinant viral vector vaccines. For example, DNA vaccines appear to be more effective in producing an antigen with a properly folded, native three-dimensional conformation and in generating a cellular immune response than are protein antigens. DNA vaccines also do not exhibit at least some of the safety problems of killed, live or virally-vectored vaccines. For example, a killed virus preparation may contain residual live viruses or may need to be mixed with reactogenic adjuvants, such as those associated with vaccine-related fibrosarcomas in cats, in order to stimulate an effective immune response. An attenuated virus may mutate and revert to a pathogenic phenotype. Viral vector vaccines genetically engineered to express a gene encoding the desired antigen may stimulate the production of antibodies that react with the virus as well; such antibodies may render futile any further attempt to use that virus as a vector, even with a different gene insert.

In contrast, DNA vaccines apparently are non-reactogenic and, if they elicit an immune response, that response is targeted against the antigen of choice.

DNA vaccines typically include a bacterial plasmid, a strong viral promoter, the gene of interest, and a polyadenylation/transcriptional termination sequence. The plasmid is typically grown in bacteria, purified, dissolved in a saline solution, and then simply injected into an animal. Current understanding of how to use DNA vaccines to generate an effective immune response, however, is not complete. Most of our understanding of the mechanisms of DNA vaccine action is derived from rodent studies. In mice, bone marrow-derived antigen-presenting cells have been shown to induce cytotoxic T lymphocyte responses following intramuscular inoculation of naked plasmid DNA. In some cases, DNA vaccination has also been shown to stimulate antigen-specific antibodies, some of which may be neutralizing antibodies. DNA vaccines have also been administered to large animals, albeit with varying degrees of success. While there are some clear examples of DNA vaccine efficacy in large animals, other studies cite relatively weak responses, requirement for large amounts of DNA, or the need for multiple immunizations. As such, it is apparent that further technology development is required to maximize DNA vaccine efficacy in humans and large animals.

Immune responses to DNA vaccination appear to vary according to the vehicle used with the DNA vaccine, the antigen expressed by the DNA vaccine, the route of administration, and the species of mammal into which the DNA vaccine is injected. Investigators have used different vehicles and/or genes encoding cytokines and other stimulatory molecules in an attempt to enhance the immune response to the antigens encoded by DNA vaccines with mixed success. Although cationic lipids have been used to deliver nucleic acids to cells *in vitro* and *in vivo*, there is no consensus in the literature about whether cationic lipids reproducibly enhance the immunogenicity of DNA vaccines. Gregoriadis et al., 1997, FEBS Letters 402, 107, reported that intramuscular (I.M.) injection of DNA encoding HBsA "entrapped" in cationic liposomes into mice elicited an enhanced immune response compared to I.M. injection of "naked" DNA encoding HBsA, whereas DNA encoding HBsA merely "complexed" with cationic lipid generated a reduced immune response compared to "naked" DNA. Ishii et al., 1997, AIDS Research and Human Retroviruses 13, 1421-1424, demonstrated enhanced

immune responses to V3 peptide following I.M., intraperitoneal (I.P.), intradermal (I.D.), intranasal (I.N.) or subcutaneous (S.Q.) administration to mice.

Other investigators, in contrast, found no enhancement of immune responses when cationic lipids were used as a vehicle for DNA vaccines in mice. For example, Davis, et al., 1997, Vaccine 15, 849, found that DNA vaccines encoding the Hepatitis B surface antigen formulated with varying amounts of cationic lipids performed no better than DNA alone in inducing a humoral response in mice. Gramzinski, et al., 1998, Molecular Medicine 4, 109, reported that Aotus monkeys administered DNA vaccines encoding HBsA either with or without cationic lipids (CELLFECTIN®, 1:1 DNA:lipid) by I.M. injection did not seroconvert. Clearly, there is no consensus regarding whether cationic lipids reproducibly act to elicit or enhance immune responses to DNA vaccines.

There also appears to be a high degree of variability of the efficacy of DNA vaccines between different routes of administration. Ishii et al, *ibid.*, for example, found in mice that I.M. and I.N. administration of DNA vaccines generated approximately equivalent immune responses, but that I.P. administration was less effective, and that I.D. and S.Q. administration routes were even less effective. Ishii et al, *ibid.*, found these differences to be consistent regardless of whether DNA was used alone or formulated with cationic lipids. Yokoyama et al, 1996, FEMS Immuno Med Microbio 14, 221-230, showed that I.V. administration of a DNA vaccine generated a better immune response than I.M. administration of the same vaccine in mice.

Taken together, these data indicate that there is a high degree of variability in the effectiveness of DNA vaccines and in the ability of cationic lipids to enhance the effectiveness of DNA vaccines both within and between species and routes of administration.

There are a number of diseases in cats which lead to significant morbidity and mortality. It would be desirable to provide novel and safe vaccines that would confer protective immunity to these diseases. That there is still a need for such vaccines is underscored not only by the association of some feline vaccines with the development of fibrosarcomas but also by the finding that I.M. administration of naked DNA encoding either human growth hormone (hGH) or rabies virus glycoprotein G into domestic cats

resulted in incomplete seroconversion, even after two immunizations (Osorio et al, 1999, Vaccine, in press). These results indicate that parenteral naked DNA vaccination efficacy in cats is inferior to results obtained in mice, and that the efficacy achieved using naked DNA in cats is not sufficient to protect cats from disease. Thus, there remains a need to provide a method to elicit and to enhance the immune response to antigen encoded by DNA vaccines in cats.

SUMMARY OF THE INVENTION

The present invention relates to a method to elicit an immune response to an antigen in a felid. This method includes the step of parenterally administering to the felid a composition comprising a nucleic acid molecule encoding the antigen in which the nucleic acid molecule is complexed with a cationic lipid. In one embodiment, this method enhances the immune response in a felid compared to a method in which a naked DNA vaccine is administered to a felid. Also provided is a method to deliver a nucleic acid molecule to a felid. This method comprises parenterally administering to the felid a composition that includes a nucleic acid molecule complexed with a cationic lipid.

DETAILED DESCRIPTION

The present invention relates to a method to elicit an immune response to an antigen in a felid. The method includes the step of parenterally administering to the felid a composition comprising a nucleic acid molecule encoding the antigen in which the nucleic acid molecule is complexed with a cationic lipid. The ability of such a method to elicit an immune response to the antigen encoded by the nucleic acid molecule is new and surprising. Until recently, the general perception of those skilled in the art was that cationic lipids did not enhance the ability of a nucleic acid molecule to elicit an immune response, compared to, for example, delivery of a naked, or unformulated, nucleic acid molecule (i.e., a nucleic acid molecule that is not complexed with, for example, a lipid or other transfection-facilitating agents). Recent studies, cited above, have provided conflicting results: although two studies in mice demonstrated that cationic lipids enhanced the ability of DNA to elicit an immune response, a third study concluded that cationic lipid-complexed DNA was no better than naked DNA at eliciting an immune response. In addition, monkeys administered a nucleic acid molecule-cationic lipid

complex did not exhibit seroconversion to the antigen encoded by the nucleic acid molecule. Furthermore, the inventors have demonstrated that while parenteral administration to a felid of a nucleic acid molecule complexed with a cationic lipid results in the felid successfully seroconverting in response to the antigen encoded by the nucleic acid molecule, intranasal administration of such a composition did not result in seroconversion. Thus, the ability to demonstrate seroconversion in cats parenterally administered a nucleic acid molecule complexed with a cationic lipid is completely unpredictable based on previous studies and, as such, is inventive.

One embodiment of the present invention is the use of a composition comprising a nucleic acid molecule encoding an antigen complexed with a cationic lipid to elicit an immune response in a felid. It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, a nucleic acid molecule, an antigen, and a cationic lipid refers to one or more nucleic acid molecules, antigens, and cationic lipids, respectively; or to at least one nucleic acid molecule, antigen, and cationic lipid, respectively. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, a member of a group that is "selected from the group consisting of" refers to one or more of members of that group, including combinations thereof.

A nucleic acid molecule of the present invention also referred to herein as a nucleic acid, can be DNA or RNA. In one embodiment, a nucleic acid molecule encodes an antigen that elicits an immune response in a felid. As such, a nucleic acid molecule can simply be a molecule that encodes such an antigen, i.e., a coding region, or the nucleic acid molecule can comprise a coding region operatively linked to a regulatory sequence. As used herein, the phrase operatively linked refers to the joining of a coding region to one or more regulatory sequences such that the coding region is expressed using such regulatory sequence(s) in a felid. Examples of such regulatory sequences include transcription control sequences and translation control sequences that can be recognized by felid cellular mechanisms in order to effect transcription and translation of a coding region. Transcription control sequences are sequences that control the initiation, elongation, and termination of transcription (e.g., promoters,

enhancers, introns, polyA sites, terminators). Translation control sequences control the initiation, elongation and termination of translation. Additional regulatory sequences include signal sequences that effect secretion of a protein from a cell and a combination of a signal sequence and a transmembrane sequence (i.e., membrane anchoring domain) that causes a protein to be partially extracellular and partially retained in the membrane and/or cytoplasm. A preferred nucleic acid molecule of the present invention is a plasmid or viral genome that includes a coding region for the desired antigen operatively linked to strong eukaryotic regulatory sequences, including a strong promoter and strong transcription termination/polyadenylation sequences. A preferred plasmid can replicate in bacteria. Procedures by which such a nucleic acid molecule is produced are known to those skilled in the art, and are disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press. Appropriate plasmids are known in the art, and may include, but are not limited to, pUC19 and BLUESCRIPT®. A preferred plasmid is pUC19. Appropriate regulatory sequences are known to those skilled in the art. For example, a suitable promoter includes, but is not limited to the cytomegalovirus immediate early promoter (CMV IE) with or without intron A, a long terminal repeat (LTR) promoter from a retrovirus, or a strong cellular promoter such as β-actin, with CMV IE with intron A being preferred. Similarly, suitable transcription termination sequences include, but are not limited to, bovine growth hormone, SV40 virus or rabbit beta-globin polyadenylation sequences, with a bovine growth hormone sequence being preferred.

A suitable antigen is any antigen that effects an immune response, and as such includes allergens and autoantigens as well as other antigens. An antigen, as used herein, can refer to the full-length antigen or any portion thereof that is capable of eliciting an immune response. Preferred antigens are those that elicit an immune response that protects an animal from disease. Examples of such antigens include, but are not limited to, a protozoan parasite antigen, a helminth parasite antigen, an ectoparasite antigen, a fungal antigen, a bacterial antigen, and a viral antigen. Examples of viral antigens include, but are not limited to, antigens from adenoviruses, caliciviruses, coronaviruses, distemper viruses, hepatitis viruses, herpesviruses, immunodeficiency viruses, infectious peritonitis viruses, leukemia viruses, oncogenic

viruses, papilloma viruses, parainfluenza viruses, parvoviruses, rabies viruses, and reoviruses, as well as other cancer-causing or cancer-related viruses. Examples of bacterial antigens include, but are not limited to, antigens from *Actinomyces, Bacillus, Bacteroides, Bordetella, Bartonella, Borrelia, Brucella, Campylobacter, Capnocytophaga, Clostridium, Corynebacterium, Coxiella, Dermatophilus, Enterococcus, Ehrlichia, Escherichia, Francisella, Fusobacterium, Haemobartonella, Helicobacter, Klebsiella*, L-form bacteria, *Leptospira, Listeria, Mycobacteria, Mycoplasma, Neorickettsia, Nocardia, Pasteurella, Peptococcus, Peptostreptococcus, Proteus, Pseudomonas, Rickettsia, Rochalimaea, Salmonella, Shigella, Staphylococcus, Streptococcus*, and *Yersinia*. Examples of fungal antigens include, but are not limited to, antigens from *Absidia, Acremonium, Alternaria, Aspergillus, Basidiobolus, Bipolaris, Blastomyces, Candida, Chlamydia, Coccidioides, Conidiobolus, Cryptococcus, Curvalaria, Epidermophyton, Exophiala, Geotrichum, Histoplasma, Madurella, Malassezia, Microsporum, Moniliella, Mortierella, Mucor, Paecilomyces, Penicillium, Phialemonium, Phialophora, Prototheca, Pseudallescheria, Pseudomicrodochium, Pythium, Rhinosporidium, Rhizopus, Scolecobasidium, Sporothrix, Stemphylium, Trichophyton, Trichosporon*, and *Xylohypha*. Example of protozoan and helminth parasite antigens include, but are not limited to, antigens from *Babesia, Balantidium, Besnoitia, Cryptosporidium, Eimeria, Encephalitozoon, Entamoeba, Giardia, Hammondia, Hepatozoon, Isospora, Leishmania*, Microsporidia, *Neospora, Nosema, Pentatrichomonas, Plasmodium, Pneumocystis, Sarcocystis, Schistosoma, Theileria, Toxoplasma*, and *Trypanosoma, Acanthocheilonema, Aelurostrongylus, Ancylostoma, Angiostrongylus, Ascaris, Brugia, Bunostomum, Capillaria, Chabertia, Cooperia, Crenosoma, Dictyocaulus, Dioctophyme, Dipetalonema, Diphyllobothrium, Diplydium, Dirofilaria, Dracunculus, Enterobius, Filaroides, Haemonchus, Lagochilascaris, Loa, Mansonella, Muellerius, Nanophyetus, Necator, Nematodirus, Oesophagostomum, Onchocerca, Opisthorchis, Ostertagia, Parafilaria, Paragonimus, Parascaris, Physaloptera, Protostrongylus, Setaria, Spirocerca, Spirometra, Stephanofilaria, Strongyloides, Strongylus, Thelazia, Toxascaris, Toxocara, Trichinella, Trichostrongylus, Trichuris. Uncinaria*, and *Wuchereria*. Examples of ectoparasite antigens include, but are not limited to, antigens

(including protective antigens as well as allergens) from fleas; ticks, including hard ticks and soft ticks; flies, such as midges, mosquitos, sand flies, black flies, horse flies, horn flies, deer flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats; ants; spiders, lice; mites; and true bugs, such as bed bugs and kissing bugs. Additional examples of suitable allergens include food, grass, weed, tree pollen, other animal and other plant allergens.

Preferred antigens include, but are not limited to, a calicivirus antigen, a coronavirus antigen, a herpesvirus antigen, an immunodeficiency virus antigen, an infectious peritonitis virus antigen, a leukemia virus antigen, a panleukopenia virus antigen, a parvovirus antigen, a rabies virus antigen, a *Bartonella* antigen, a *Yersinia* antigen, a *Dirofilaria* antigen, a *Toxoplasma* antigen, a tumor antigen, a flea antigen, a flea allergen, a midge antigen, a midge allergen, a mite antigen, a mite allergen, a ragweed allergen, a ryegrass allergen, a cat allergen, a dog allergen, a Bermuda grass allergen, a Johnson grass allergen, or a Japanese cedar pollen allergen. Particularly preferred antigens include a rabies virus glycoprotein G antigen; heartworm PLA2, P39, P4, P22U, Gp29, astacin, cysteine protease, macrophage migration inhibitory factor, venom allergen, TPX-1, TPX-2, transglutaminase, ankyrin, asparaginase, calreticulin, cuticulin, and aromatic amino aid decarboxylase antigens; flea serine protease, cysteine protease, aminopeptidase, serpin, carboxylesterase, juvenile hormone esterase, chitinase, epoxide hydrolase, ecdysone, ecdysone receptor, and ultraspiracle protein antigens; flea salivary antigens; *Yersinia* F1 and V antigens; and *Toxoplasma gondii* antigens such as those disclosed in PCT Patent Publication No. WO 99/32633, published July 1, 1999, by Milhausen et al. Additional examples of suitable and preferred allergens are disclosed in U.S. Patent No. 5,945,294, issued August 31, 1999, by Frank et al. (US 5,945,294); U.S. Patent No. 5,958,880, issued September 28, 1999, by Frank et al. (US 5,958,880); PCT Patent Publication No. WO 98/45707, published October 15, 1998, by Frank et al. (WO 98/45707); and PCT Patent Publication No. WO 99/38974, published August 5, 1999, by Weber et al. (WO 99/38974).

One embodiment of the present invention is a composition comprising a nucleic acid molecule-cationic lipid complex that further comprises a heterologous nucleic acid molecule encoding an immunomodulator. Such an immunomodulator-encoding nucleic

acid molecule can be contained within the same nucleic acid molecule encoding the antigen of the present invention, or can exist as a separate nucleic acid molecule, which can be on the same or separate plasmid or viral genome. The present invention also includes Suitable immunomodulators include compounds that enhance certain immune responses as well as compounds that suppress certain immune responses. Compounds that enhance the immune response include compounds that preferentially enhance humoral immunity as well as compounds that preferentially enhance cell-mediated immunity. Suitable compounds can be selected depending on the desired outcome. Suitable immunomodulators include, but are not limited to, cytokines, chemokines, superantigens, co-stimulatory molecules, adhesion molecules, and other immunomodulators as well as compounds that induce the production of such immunomodulators. Examples of such compounds include, but are not limited to, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), erythropoietin (EPO), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 18 (IL-18), interferon gamma, interferon gamma inducing factor I (IGIF), transforming growth factor beta (TGF-β), RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory proteins (e.g., MIP-1 alpha and MIP-1 beta), Leishmania elongation initiating factor (LEIF), B7-1, B7-2, CD40, CD40 ligand, ICAM-1, and VCAM.

A composition of the present invention includes a cationic lipid complexed with a nucleic acid molecule encoding an antigen in order to elicit or enhance an immune response to the antigen. As used herein, a cationic lipid is a lipid which has a cationic, or positive, charge at physiologic pH. Cationic lipids can have a variety of forms, including liposomes or micelles. Whether a cationic lipid occurs primarily as a liposome or a micelle can be manipulated by methods known in the art; for example, a freezing and thawing of cationic lipids in aqueous solution will encourage formation of liposomes, rather than micelles. A nucleic acid molecule complexed with a cationic lipid may also be referred to as a nucleic acid molecule-cationic lipid complex, a

lipoplex or a complex of the present invention. A complex of the present invention that elicits an immune response is a complex of a nucleic acid molecule which encodes an antigen with a cationic lipid. As used herein, the term complexed with, which is equivalent to complexed to, refers to any method by which a nucleic acid molecule interacts (e.g. binds, comes into contact with a cationic lipid.) Such an interaction can include, but is not limited to encapsulation of a nucleic acid molecule into a cationic liposome, association of a nucleic acid molecule and cationic lipid characterized by non-covalent, ionic charge interactions, and other types of associations between nucleic acid molecules and cationic lipids known by those skilled in the art. It is preferred that cationic lipids have a cationic group, such as a quaternary amine group, and one or more lipophilic groups, such as saturated or unsaturated alkyl groups having from about 6 to 30 carbon atoms. Cationic lipid compositions suitable for use in the present invention include lipid compositions comprised of one type of lipid, or lipid compositions comprised of more than one type of lipid. If there is more than one type of lipid present in a lipid composition, it is necessary that the overall net charge of the lipid composition is cationic, i.e. positive; however, as long as the overall net charge of the lipid composition is cationic, individual lipid types may be neutral or even anionic in charge. A composition of the present invention includes a cationic lipid that is suitable in accordance with the present invention. Cationic lipids suitable for use in the present invention include commercially available cationic lipids, for example DOTMA, available under the trademark name of LIPOFECTIN®, available from Life Technologies Inc., (LTI), Gaithersburg, MD and DDAB, available from Boehringer-Mannheim, Indianapolis, IN. In addition, suitable cationic lipids can be synthesized as described in the literature; see, for example, Felgner et al., 1987, PNAS 84 7413-7417 regarding the preparation of DOTAP; Douar et al, 1996, Gene Ther 3(9), 789-796 regarding the preparation of Lipid 67; Wheeler et al., 1996, Biochim Biophys Acta 1280(1), 1-11 regarding the preparation of DMRIE; McLean et al., 1997 Am J Physiol 273, H387-404 regarding the preparation of DOTIM; and Hofland et al., 1997, Pharm Res 14(6), 742-749 regarding the preparation of DOSPA. Other suitable cationic lipid compounds are described in the literature. See, for example, Stamatatos et al., 1988, Biochemistry 27, 3917-3925 and Eibl, et al., 1979, Biophysical Chemistry 10, 261-271.

Preferred cationic lipids include the class of lipids known as tetramethyltetraalkyl spermine analogs, described by McCluskey et al., (1998), *Antisense and Nucleic Acid Drug Development*, vol. 8, pp 401-414. Lipids of this type include tetramethyltetralaurylspermine, tetramethyltetramyristylspermine, tetramethyltetrapalmitoylspermine, and tetramethyltetraoleoylspermine. The following lipids, obtained from LTI are of the tetramethyltetraalkyl spermine class, with the alkyl groups containing fatty acid chains of length longer than oleic acid. These lipids are denoted as LTI lipids 4251-781-1, 4251-106-3, 4518-52, D304-200, 4521-52-3, 4251-106-4, 4251-781-2, 4518-53, 4518-31, 4519-30, 4519-34, and 2518-111. Preferred cationic lipids include LTI lipid 4251-781-1, LTI lipid 4251-106-3, and LTI lipid 4518-52. In one embodiment, tetramethyltetraalkyl spermine lipids are formulated with a neutral lipid, such as dioleylphosphatidyl-ethanolamine (DOPE).

A nucleic acid molecule-cationic lipid complex can be formed by using techniques known to those skilled in the art, examples of which are described in the Examples section. A complex can be formed, for example, by adding a cationic lipid solution to a nucleic acid molecule, preferably an endotoxin-free nucleic acid molecule, at concentrations appropriate for the present invention, and mixing, for example by pipetting. Preferable nucleic acid molecule-to-cationic lipid ratios are from about 10:1 weight nucleic acid molecule: weight cationic lipid, (e.g. microgram (μg) nucleic acid molecule to μg cationic lipid) to about 1:10 weight nucleic acid molecule: weight cationic lipid. More preferable are ratios from about 1:2 weight of nucleic acid molecule: cationic lipid to about 4:1 weight of nucleic acid molecule: cationic lipid. In a preferred embodiment, the nucleic acid molecule-cationic lipid complex is incubated at room temperature for about 30 minutes before administration. A nucleic acid molecule-cationic lipid complex can be dehydrated and rehydrated using techniques known to those skilled in the art; for example, the complex can be frozen in liquid nitrogen and lyophilized at 150 milliTorr, then reconstituted in solution for injection.

A dose of a nucleic acid molecule-cationic lipid complex to administer to a cat can be reported as the amount of nucleic acid molecule administered to a cat. A preferred dose of a nucleic acid molecule-cationic lipid complex to administer to a cat includes from at least one nanogram (ng) of nucleic acid to about 10 milligram (mg) of

nucleic acid molecule. More preferred is a dose range that includes from about 1 μg nucleic acid molecule to about 1 mg of nucleic acid molecule. Particularly preferred is a dose ranging from about 75 μg of a nucleic acid molecule to about 300 μg of a nucleic acid molecule.

A nucleic acid molecule-cationic lipid complex composition of the present invention can be formulated in an excipient that the animal to be treated can tolerate. As such, the present invention includes administration of a composition comprising a nucleic acid molecule-cationic lipid complex, wherein the composition further comprises an excipient. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, mannitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

In one embodiment of the present invention, the nucleic acid molecule-cationic lipid complex can also include an adjuvant and/or a carrier. One advantage of a nucleic acid molecule-cationic lipid complex is that adjuvants and carriers are not required to produce a composition that administration thereof will elicit an immune response. However, it should be noted that use of adjuvants or carriers is not precluded by the present invention. Adjuvants are typically substances that generally enhance the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, other bacterial cell wall components; aluminum-based salts; calcium-based salts; silica; polynucleotides; toxins, such as cholera toxin; toxoids, such as cholera toxoid; serum proteins; other viral coat proteins; other bacterial-derived preparations; block copolymer adjuvants, such as Hunter's Titermax™ adjuvant (Vaxcel™, Inc. Norcross, GA); Ribi adjuvants (available from Ribi ImmunoChem

Research, Inc., Hamilton, MT); and saponins and their derivatives, such as Quil A (available from Superfos Biosector A/S, Denmark). Carriers are typically compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release formulations, biodegradable implants, liposomes, bacteria, viruses, oils, esters, and glycols.

An immune response to an antigen includes a humoral, i.e. antibody, response to that antigen and/or a cell-mediated response to that antigen. Methods to measure an immune response are known to those skilled in the art; examples of such methods are disclosed herein. If one or both types of immune response are present, they may protect the felid from disease caused, for example, by the agent from which the antigen was derived. In accordance with the present invention, the ability of an antigen derived from a disease-causing agent to protect an animal from a disease caused by that disease-causing agent or a cross-reactive agent refers to the ability of a nucleic acid molecule-cationic lipid complex of the present invention to treat, ameliorate and/or prevent disease caused by the disease-causing agent or cross-reactive agent, preferably by eliciting an immune response against the antigen derived from the disease-causing agent. It is to be noted that an animal may be protected by a composition of the present invention even without the detection of a humoral or cell-mediated response to the antigen. Protection can be measured by methods known to those skilled in the art, such as by challenging an animal with the agent against which the animal has mounted a putative immune response. In certain cases, the antibody titer of an animal can be used to demonstrate protection. For example, it is known that animals that elicit an antibody response against a rabies glycoprotein G antigen are protected if their sera exhibits a rapid focus fluorescent inhibition test (RFFIT) titer of rabies virus neutralizing antibodies of greater than 1:5. As used herein, an animal that elicits an immune response to an antigen is an animal that has been immunized with that antigen.

The biological mechanism for eliciting and/or enhancing an immune response by the use of a nucleic acid molecule-cationic lipid complex composition of the present invention has not been elucidated, but, without being bound by theory, the inventors believe that the mechanism is likely related to the ability of these compositions to protect DNA from nuclease attack, to facilitate the transfection of both muscle cells and

professional antigen presenting cells (APC) *in vivo*, to increase levels of expression in transfected cells, and/or to distribute DNA to lymphoid organs.

A felid, as used herein, is a member of the family Felidae. Examples of felids include domestic cats, wild cats, and zoo cats. Examples of cats, include, but are not limited to, domestic cats, lions, tigers, leopards, panthers, cougars, bobcats, lynx, jaguars, cheetahs, and servals. A preferred cat to immunize is a domestic cat. The term cat(s) and felid(s) are used interchangeably herein.

As used herein, parenteral administration means administration not through the alimentary canal (e.g. oral administration), but rather by injection through some other route, including but not limited to routes such as subcutaneous, intramuscular (I.M.), intravenous (I.V.), intraperitoneal (I.P.), intradermal (I.D.), intraorbital, intracapsular, intraspinal, and intrasternal. Parenteral administration includes, but is not limited to, administration by any route that includes use of a needle to insert material into the body. Parenteral administration also includes uses of devices other than a syringe and needle to insert material through the skin and or mucosal surfaces into the body, including but not limited to the BIOJECTOR®, POWDERJECT, and MEDIJECT® needleless injection systems. A preferred route of administration includes intramuscular administration using a needle and syringe.

Acceptable protocols to administer therapeutic compositions in an effective manner include individual dose size, number of doses, and frequency of dose administration. Typically, the first administration of a composition intended to elicit an immune response is called the primary (or prime) administration, also known as the pre-boost. Additional administrations intended to "boost" or increase an immune response to an antigen are termed booster administrations. Determination of a protocol to elicit an immune response in a cat using a nucleic acid molecule-cationic lipid complex of the present invention can be accomplished by those skilled in the art. In one embodiment of the present invention, a nucleic acid molecule encoding a desired antigen complexed with cationic lipid need only be administered once by a route appropriate to the present invention (e.g. parenteral) to stimulate an immune response against the antigen. In a preferred embodiment, such an administration protects the felid from the agent from

which the antigen was derived or from an agent against which the immune response is cross-protective.

In one embodiment, administration of a complex of the present invention to a felid in order to elicit an immune response actually enhances the immune response generated by the felid as compared to the immune response generated upon administration of a naked DNA vaccine to a felid, wherein the naked DNA vaccine consists essentially of a naked DNA molecule; i.e., a DNA molecule that is not complexed with lipids. Finding that a complex of the present invention enhances an immune response is surprising both in view of the conflicting studies known to those skilled in the art as described herein as well as in view of the studies described in more detail in the Examples, in which administration of naked DNA vaccines to cats elicited immune responses in only some cats within each group, or population, tested, whereas administration of a complex of the present invention could result in up to 100% seroconversion of all cats in a population tested. As used herein, enhancement of the immune response can include increasing the amount, or titer, of antibody elicited by a complex of the present invention that encodes an antigen to the desired antigen and/or agent from which the antigen was derived as compared to the titer of antibody generated by a naked DNA vaccine that encodes the same antigen. In one embodiment, such an enhancement can be induction of no antibody titer with a naked DNA vaccine to induction of a protective antibody titer with a complex of the present invention. Enhancement of an immune response can also refer to augmentation of the cell-mediated response to the antigen and/or agent encoded by a complex of the present invention as compared to the response generated by a naked DNA vaccine encoding the same antigen. Enhancement of immune response can also include conferring or augmenting protection from disease by a complex of the present invention compared to the protection, if any, conferred by a naked DNA vaccine encoding the same antigen. In one embodiment, enhancement of the immune response includes increasing the likelihood of a cat seroconverting in response to antigen encoded by a complex of the present invention in comparison to the likelihood of the cat responding to the same antigen encoded by a naked DNA vaccine. In other words, in a group of cats being vaccinated with a complex of the present invention, a greater number of cats will

seroconvert in response to antigen encoded by the complex rather than to the same antigen encoded by a naked DNA vaccine. Preferably, the likelihood that a cat will seroconvert when administered a single dose of a complex of the present invention that encodes an antigen is at least about 50%, preferably at least about 75%, more preferably at least about 90% and even more preferably at least about 100%. In the case where a primary and booster administration of the complex is administered, the likelihood that a cat will seroconvert is preferably at least about 75%, more preferably at least about 90%, and even more preferably at least about 100%.

The present invention includes a method to administer a nucleic acid molecule to a felid. The method includes the step of parenterally administering a composition comprising said nucleic acid molecule complexed with a cationic lipid. Such a nucleic acid molecule can encode either a protein or a RNA molecule. In one embodiment, the nucleic acid molecule encodes a protein or RNA molecule that, when expressed at an appropriate level, has a protective effect upon the cat. As used herein, a protein refers to a full-length protein or any portion thereof that is at least about 5 amino acids in length and has a useful function, including, but not limited to, ability to elicit an immune response, elicit an immunomodulatory effect (e.g., an immunomodulator that stimulates or reduces the immune response), effect gene therapy, effect enzyme activity, or otherwise effect cell division, differentiation, development and cell death. As used herein, a RNA molecule refers to any RNA species that can be encoded by a nucleic acid molecule, including, but not limited to antisense RNA, a molecule capable of triple helix formation, a ribozyme, or other nucleic acid-based drug compound. As such, any protein or RNA molecule that can be expressed at an appropriate level in a cat, which protects a cat from disease, would be included in this invention. Diseases from which to protect a felid include, but are not limited to, infectious diseases, genetic diseases, oncological diseases, and other metabolic diseases, including diseases that lead to abnormal cell growth, degenerative processes, and immunological defects. Compositions of the present invention can protect animals from a variety of diseases including, but not limited to, allergies, arthritic diseases, autoimmune diseases, cancers, cardiovascular diseases, graft rejection, hematopoietic disorders, immunodeficiency diseases, immunoproliferative diseases, immunosuppressive disorders, infectious

diseases, inflammatory diseases, jaundice, septic shock, and other immunological defects, as well as other genetic or metabolic defects. Methods to produce and use a composition comprising any nucleic acid molecule of the present invention complexed with any cationic lipid of the present invention are as described herein.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

EXAMPLES

Example 1

This Example demonstrates the production of a nucleic acid molecule of the present invention.

A nucleic acid molecule encoding human growth hormone (hGH) was constructed using plasmid pHGH107 (available from American Type Culture Collection, Manassis, VA), which encodes hGH amino-acids 1-191, as a polymerase chain reaction (PCR) template. The hGH open reading frame was amplified by PCR using Pfu DNA polymerase (available from Stratagene, La Jolla, CA) and the following forward and reverse primers: 5' TTCCCAACTATACCACTATCTCGTCTA 3' (SEQ ID NO:1) and 5' CTAGAAGCCACAGCTGCCCTCCACAGAG 3' (SEQ ID NO:2). The PCR product containing the sequence encoding the mature hGH product was ligated into the *Nae*I site of a plasmid containing the human cytomegalovirus immediate early promoter, a translation control sequence, a sequence encoding the signal peptide coding sequence from human tissue plasminogen activator, and a bovine growth hormone poly A sequence. The expression of hGH from this plasmid was confirmed following transfection of cells *in vitro* and was detected using a chemiluminescence assay kit (available from Nichols Institute Diagnostics, San Juan Capistrano, CA).

A nucleic acid molecule encoding the rabies virus glycoprotein G was described previously and contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence. See Osorio, et al. (1999) Vaccine, in press.

Example 2

This Example describes the production of a nucleic acid molecule-cationic lipid complex of the present invention.

Endotoxin-free nucleic acid molecules encoding hGH or rabies glycoprotein G were prepared using a commercial kit (Qiagen, Inc., Valencia, CA) and the resulting nucleic acid molecules were dissolved in endotoxin-free 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 2 mg per milliliter (ml) to form a hGH nucleic acid molecule solution and a rabies gG nucleic acid molecule solution, respectively. Cationic lipids 4251-106-3 (also known as 106-3), 4251-781-1 (also known as 781-1), and 4518-52 were obtained from Life Technologies, Inc. (LTI), Gaithersburg, MD. A nucleic acid molecule-cationic lipid complex was formed by adding 250 μl of the respective cationic lipid solution to 250 μl of the respective nucleic acid molecule solution, followed by immediate mixing by pipetting. The concentrations of the cationic lipid solutions and of the nucleic acid molecule solutions used were adjusted to give the desired amounts and ratios of nucleic acid molecules to cationic lipids described elsewhere in the Examples. The mixture was incubated at room temperature for 30 minutes before administration. For dehydration and rehydration of a nucleic acid molecule-cationic lipid complex, the complex was frozen in liquid nitrogen and lyophilized at 150 mTorr, then reconstituted in the original volume of sterile water for injection.

Example 3

This Example describes a method for administering a nucleic acid molecule-cationic lipid complex of the present invention to a felid.

Primary and booster administrations of nucleic acid molecule-cationic lipid complexes prepared as described in Example 2 were injected intramuscularly into the semitendinosus or semimembranosus muscle of domestic cats. Each dose was divided into two equal portions and administered bilaterally into each leg. Sera samples were collected every 10 days for antibody determination.

Example 4

This Example describes methods to measure immune responses generated in response to the administration of nucleic acid molecule-cationic lipid complexes of the present invention.

Antibody responses specific for hGH were determined by ELISA. Briefly, ELISA plate wells were coated with 0.4 micrograms (μg) hGH protein per well (hGH protein available from Genzyme Diagnostics, San Carlos, CA) and incubated overnight

at 4°C. Unbound antigen was aspirated and the plate was blocked with 2% skimmed milk for 1 hour at 37°C . ELISA plates were washed 3 times with TBS-Tween (150 milliMolar (mM) NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% TWEEN-20) and serially diluted sera samples from vaccinated cats were added and incubated at 37°C for 1 hour. Plates were washed 3 times with TBS-Tween. A biotin conjugated monoclonal anti-cat IgG (1:30,000) (available from Sigma-Aldrich, St. Louis, MO), was added and incubated for 1 hour at 37°C, followed by the addition of EXTRAVIDIN®-horseradish peroxidase diluted 1:1000, available from Sigma-Aldrich, St. Louis, MO. After a final incubation at 37°C, for 1 hour, the plates were washed and an o-phenylenediamine dihydrochloride substrate solution, available from Sigma-Aldrich, was added and the plates incubated at room temperature for 30 minutes for color development. The plates were read at 450 nm.

Rabies virus-specific neutralizing antibody response were determined using the Rapid Fluorescent Focus Inhibition test (RFFIT) at the Department of Veterinary Diagnostics, Kansas State University.

T cell proliferation assays were carried out in the following manner. Heparinized blood samples were collected from cats a week after administration of a booster injection as described in Example 5. The lymphocytes were isolated from the blood samples using a percoll gradient (Sigma Chemicals, St Louis, MO). The isolated lymphocytes were resuspended in RPMI 1640 (Sigma Chemical) containing 5% normal cat serum, 2 mM L-glutamine (Life Technologies, Bethesda, MD), 1 mM sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Life Technologies), 5 μg/mL gentamycin (Sigma Chemical), 0.1 mM MEM non-essential amino acids (Life Technologies), and 1% essential amino acids (Life Technologies) plated at a density of 2×10^5 cells/well and treated with various concentrations of recombinant human growth hormone (hGH) (Genzyme Diagnostics, Boston, MA) for a total of 3 or 5 days. Each group of cell samples contained a negative control (media alone) and a positive control (Concanavalin A, Sigma Chemicals). Cells were pulsed at time of measurement with 0.5 μCurie of tritiated thymidine (ICN Pharmaceuticals) per well. T cells that were specific for hGH proliferated in response to added hGH and incorporated the tritiated thymidine into their DNA. The amount of incorporated tritium was counted 16 to 18

hours post-pulse in a scintillation counter. Data was reported as the stimulation index, which was derived by dividing the counts per minute obtained from the samples divided by the counts per minute obtained from the negative control.

Example 5

This Example compares the immune response elicited using a nucleic acid molecule encoding hGH complexed with either LTI lipid 781-1 or LTI lipid 106-3 to the immune response elicited using a naked DNA vaccine encoding hGH in cats.

The hGH nucleic acid molecule was complexed with LTI lipid 781-1 at a lipid-to-DNA ratio (μg:μg) of 0.5:1.0, and formulated with LTI lipid 106-3 at a lipid-to-DNA ratio of 1:1, as described in Example 2. The naked DNA vaccine consisted of the hGH nucleic acid molecule prepared as described in Example 2 dissolved in saline.

A total of 12 cats were divided into three vaccine groups as follows:

Group 1 (naked DNA): Two injections, spaced 8 weeks apart, of 300 μg of naked hGH nucleic acid molecule in 500 μl saline.

Group 2 (LTI lipid 781-1): Two injections, spaced 8 weeks apart, of 300 μg hGH nucleic acid molecule complexed with 150 μg cationic lipid.

Group 3 (LTI lipid 106-3): Two injections, spaced 8 weeks apart, of 300 μg hGH nucleic acid molecule complexed with 300 μg cationic lipid.

At day 54 post injection, the cats were boosted with another injection of the appropriate cationic lipid-DNA mixture. At day 111, cats were boosted again, and at day 119, T-cell proliferation assays were performed as described. A T-cell stimulation index of 2 is taken as the cutoff and values below 2 are considered non-responsive.

Sera samples were collected from cats following the primary and booster administrations of complex as described in Example 3 and were assayed for hGH specific antibody responses by ELISA. Endpoint ELISA titers are shown in Table 1. The lowest sera titers measured were 1:40. Therefore, negative titers are expressed as <1:40.

Table 1. hGH antibody titers of sera samples collected from cats administered a naked DNA vaccine or a complex of the present invention encoding hGH

cat #	Formulation	Titer at day 54 (post prime)	Titer at day 64 (post boost)	T-cell stimulation index
1	Naked DNA	<1:40	<1:40	9.1
2	Naked DNA	<1:40	<1:40	2.4
3	Naked DNA	<1:40	1:1125	12.7
4	Naked DNA	<1:40	<1:40	11.3
		geometric mean=40	geometric mean=92	
5	DNA + lipid 781-1	<1:40	<1:40	1.6
6	DNA + lipid 781-1	1:160	1:10,240	20.2
7	DNA + lipid 781-1	1:2312	1:7762	3.7
8	DNA + lipid 781-1	1:233	1:21,183	8.9
		geometric mean=242	geometric mean=2865	
9	DNA + lipid 106-3	1:1076	1:19,000	24.3
10	DNA + lipid 106-3	1:316	1:19,135	65.5
11	DNA + lipid 106-3	<1:40	<1:40	18.2
12	DNA + lipid 106-3	1:125	1:8693	2.6
		geometric mean=203	geometric mean=3353	

The results in Table 1 indicate that there was no seroconversion in any of the four cats administered a single inoculation of 300 μg of the naked hGH nucleic acid molecule. Moreover, following the booster administration, only one of the four cats in the naked DNA vaccine group developed an hGH-specific antibody response. In contrast to the naked DNA vaccine group, 75% of the cats (i.e., 3 of 4 cats) cats in each of the two lipid groups developed detectable titers following the primary administration of complex, and these responses went up markedly following the booster administration of complex.

T-cell proliferation, measured by the T cell stimulation index, indicates that all treatments, including treatment with DNA alone, appeared to activate cell-mediated immunity. Treatment with a complex of DNA and lipid 106-3 appears to work better for

stimulating T cell proliferation in cats than did naked DNA alone or DNA complexed with lipid 781-1.

Example 6

This Example compares immune responses elicited using a nucleic acid molecule encoding rabies glycoprotein G complexed with several cationic lipids of the present invention to the immune response elicited using a naked DNA vaccine encoding rabies glycoprotein G in cats.

This example compared the abilities of the following compositions to elicit an immune response against rabies glycoprotein G (rabies G) in cats: a naked DNA vaccine consisting of the rabies G nucleic acid molecule; and complexes between the rabies G nucleic acid molecule and one of the following cationic lipids: LTI lipid 106-3, LTI lipid 781-1, or LTI lipid 4518-52, each at a variety of DNA:lipid ratios. Also tested was a complex that had been dehydrated by lyophilization and rehydrated prior to administration. Each of the compositions was produced as described in Example 2. All cats received two intramuscular injections as described in Example 3, spaced four weeks apart. The following groups of 4 cats each were tested:

Group 1: Naked DNA, 300 μg rabies G vector

Group 2: 300 μg lipid 781-1 + 300 μg rabies G vector

Group 3: 150 μg lipid 781-1 + 300 μg rabies G vector

Group 4: 75 μg lipid 781-1 + 300 μg rabies G vector

Group 5: 600 μg lipid 106-3 + 300 μg rabies G vector

Group 6: 300 μg lipid 106-3 + 300 μg rabies G vector

Group 7: 150 μg lipid 106-3 + 300 μg rabies G vector

Group 8: 300 μg lipid 4518-52 + 300 μg rabies G vector

Group 9: 300 μg lipid 106-3 + 300 μg rabies G vector (lyophilized and rehydrated)

Group 10: 75 μg lipid 106-3 + 75 μg rabies G vector.

Group 1 served as a control group to demonstrate immunogenicity of the naked DNA vaccine. Groups 2-4 were designed to determine if differences in the lipid-to-DNA ratio were important for lipid 781-1. Similarly, groups 5-7 were designed to determine if differences in the lipid-to-DNA ratio were important for lipid 106-3.

Group 8 was included to examine the efficacy of LTI lipid 4518-52. Group 9 was included to determine if lyophilization and rehydration of lipid:DNA complexes would improve cationic lipid vaccine efficacy in cats as previously demonstrated in mice by Gregoriadis, *ibid.* Finally, group 10 was included to determine is less than 300 μg of DNA could be used without affecting the ability of lipid 106-3 to enhance the ability of cats to elicit an immune response.

Rabies virus-specific neutralizing antibody activity was measured in the sera of all cats before and after the booster administration by RFFIT. Sera dilutions tested ranged from 1:5 to 1:174,693. Negative responses are listed as a titer of <1:5 while responses that are stronger than the final dilution tested are indicated by the ">" sign. Injections were made intramuscularly. It is known to those skilled in the art that an anti-rabies G antibody titer of 1:5 or greater, as measured by RFFIT, is protective. Results from these studies are shown in Table 2.

Table 2. Rabies G antibody titers of sera samples collected from cats administered a naked DNA vaccine or a complex of the present invention encoding rabies G.

Group 1

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHR5	Naked DNA (300 μg rabies G)	<1:5	1:25
BWM3	Naked DNA (300 μg rabies G)	<1:5	<1:5
3042	Naked DNA (300 μg rabies G)	<1:5	1:1400
ABO2	Naked DNA (300 μg rabies G)	<1:5	<1:5

Group 2

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHH1	300 μg DNA + 300 μg lipid 781-1	1:7000	1:167,449
3102	300 μg DNA + 300 μg lipid 781-1	1:1800	1:167,449
S72	300 μg DNA + 300 μg lipid 781-1	<1:5	1:50
QJB1	300 μg DNA + 300 μg lipid 781-1	<1:5	1:230

Group 3

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHN4	300 μg DNA + 150 μg lipid 781-1	<1:5	1:1400
QIN5	300 μg DNA + 150 μg lipid 781-1	1:2200	1:42,724
QGN5	300 μg DNA + 150 μg lipid 781-1	1:625	1:113,264
QHG1	300 μg DNA + 150 μg lipid 781-1	<1:5	1:7000

Group 4

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHR1	300 μg DNA + 75 μg lipid 781-1	<1:5	1:50
QIN2	300 μg DNA + 75 μg lipid 781-1	1:280	1:5100
QGR5	300 μg DNA + 75 μg lipid 781-1	1:2400	1:6000
ACN1	300 μg DNA + 75 μg lipid 781-1	<1:5	1:125

Group 5

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3603	300 μg DNA + 600 μg lipid 106-3	1:7000	1:174,693
BNJ2	300 μg DNA + 600 μg lipid 106-3	<1:5	1:1800
ZAH1	300 μg DNA + 600 μg lipid 106-3	1:5100	1:159,751
S203	300 μg DNA + 600 μg lipid 106-3	1:6300	1:67,491

Group 6

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3525	300 μg DNA + 300 μg lipid 106-3	1:280	1:45,668
BNJ1	300 μg DNA + 300 μg lipid 106-3	1:125	1:6800
BMX2	300 μg DNA + 300 μg lipid 106-3	<1:5	1:6800
S197	300 μg DNA + 300 μg lipid 106-3	>1:167,449	1:6800

Group 7

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3553	300 μg DNA + 150 μg lipid 106-3	1:1100	1:53,888
BN13	300 μg DNA + 150 μg lipid 106-3	<1:5	1:3125
E490	300 μg DNA + 150 μg lipid 106-3	1:25	1:6800
S192	300 μg DNA + 150 μg lipid 106-3	1:40	1:6000

Group 8

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3541	300 μg DNA+300 μg lipid 4518-52	1:45	1:7000
BNH4	300 μg DNA+300 μg lipid 4518-52	1:1200	1;142,858
BLR1	300 μg DNA+300 μg lipid 4518-52	1:280	1:7000
S189	300 μg DNA+300 μg lipid 4518-52	>1:7000	1:159,751

Group 9

cat #	Formulation	Titer Pre-boost	Titer Post-boost
	DNA + lipid 106-3, dehyd &.rehyd[1]	1:2700	1:142,858
BNF4	DNA + lipid 106-3, dehyd &.rehyd[1]	1:170	>1:167,449
E457	DNA + lipid 106-3, dehyd &.rehyd[1]	1:1400	1:8,125
S186	DNA + lipid 106-3, dehyd &.rehyd[1]	1:45	1:3,125

[1]300 μg rabies G DNA + 300 μg lipid 106-3/dehydrated and rehydrated by the method of Gregoriadis, et al., *ibid.*

Group 10

cat #	Formulation	Titer Pre-boost	Titer Post-boost
BMC1	DNA, 75 μg + lipid 106-3, 75 μg	1:1800	1:6000
E451	DNA, 75 μg + lipid 106-3, 75 μg	1:3125	1:38,206
QNV1	DNA, 75 μg + lipid 106-3, 75 μg	1:360	1:34,600
ZAF1	DNA, 75 μg + lipid 106-3, 75 μg	1:440	1:5,400

Geometric Mean Titers for each group, pre-boost and post boost, for each group

Group	Formulation	mean titer, pre-	mean titer, post-
1	Naked DNA (300 μg)	<5	30.6
2	DNA, 300 μg + lipid 781-1, 300 μg (1:1)	133	4238
3	DNA, 300 μg + lipid 781-1, 150 μg (1:0.5)	77	14,756
4	DNA, 300 μg + lipid 781-1, 75 μg (1:0.25)	64	661
5	DNA, 300 μg + lipid 106-3, 600 μg (1:2)	1029	42,910
6	DNA, 300 μg + lipid 106-3, 300 μg (1:1)	413	10,409
7	DNA, 300 μg + lipid, 106-3, 150 μg (1:0.5)	48	9104
8	DNA, 300 μg + lipid 4518-52, 300 μg (1:1)	570	32,518
9	DNA, 300 μg + lipid, 106-3, 300 μg dehyd& rehyd[1]	412	49,159
10	DNA, 75 μg + lipid 106-3, 75 μg (1:1)	972	14,385

The data presented in Table 2 support the following conclusions: (1) in the cats receiving the naked DNA vaccine, no seroconversion was observed following the primary administration of vaccine immunization. In contrast, all of the cats receiving a nucleic acid molecule - cationic lipid complex of the present invention exhibited seroconversion after the booster administration, and at least 50% of the cats seroconverted per group after the initial administration of the complex. The best seroconversion was seen in groups 8, 9, and 10 in which 100% seroconversion was observed following the primary administration of complex. These results (0% seroconversion in group 1 and 100% seroconversion in groups 8-10 following the primary administration) were statistically significant by Fisher's exact test ($P<0.05$). (2) Following the booster administration, all nine groups that were administered a complex of the present invention exhibited stronger responses than the naked DNA vaccine control group. Despite the small number of cats in each group, statistically significant enhancement by Student's t test was observed in groups 5 and 9 as compared to group 1, i.e. naked DNA vaccine. (3) Varying the ratio of lipid-to-DNA did not have significant impact on the degree of enhancement (groups 2-4 and 5-7). (4) Dehydration and rehydration of the lipid:DNA complexes (lipid 106-3) prior to inoculation resulted

in 100% seroconversion following the primary administration and very strong responses in all cats following the boost (group 9). (5) Reducing the DNA dosage to 75 μg from 300 μg did not result in any loss of the enhancement potential since 100% seroconversion was observed after the primary administration of the complex and very strong responses were observed in all cats post-boost (group 10).

Example 7. Measuring luciferase expression in cat muscle.

Muscle and lymph node tissues were dissected and removed from the thigh of a sacrificed cat, see Example 3. The tissues were quick frozen on dry ice, and ground to a powder in liquid nitrogen. Ground frozen tissue was resuspended in 1X cell culture lysate reagent (25 mM Tris-Phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2 diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). After lysis, the cell debris was removed by centrifugation and supernatant was used in the following assay. An aliquot of the supernatant was mixed with Luciferase Assay Reagent, (Promega, Madison, WI). The mixture was placed in a Turner Designs Luminometer TD-20/20, (Promega), and the light emitted was measured for 15 seconds. The standard used to calibrate the assay was the recombinant firefly luciferase QUANTILUM™, (Promega).

Example 8. Comparison of expression of a DNA plasmid, formulated with and without LTI lipid 106-3, in the cat muscle.

In this example, evidence for increased antigen expression in the muscle upon formulation with lipid 106-3 was observed in an experiment in which 300 μg of a plasmid vector encoding luciferase was injected into each semimembranosus muscle (inner thigh) of a cat, one muscle receiving DNA complexed with lipid, and one muscle receiving naked DNA. In the case of DNA formulated with lipid 106-3, 300 μg of DNA was formulated with 300 μg of lipid 106-3. Specifically, the right thigh of the cat was injected with DNA alone; the left thigh was injected with DNA formulated with lipid 106-3. After 48 hours, the cat was sacrificed, the muscles were dissected and luciferase activity was measured as described in Example 7. Table 3 shows the luciferase assay standard curve used for this experiment, and Table 4 shows luminometer measurements for each dissected tissue in the cat.

Table 3. Luciferase assay standard curve

Sample	Luminometer readings
Blank	0.069
Positive control	332
2.5 μg standard	387.9
250 nanogram(ng) standard	54.07
25 ng standard	9.817
2.5 ng standard	1.909
250 picogram (pg) standard	0.431
25 pg standard	0.189

Table 4. Luminometer readings for each dissected muscle

muscle tested	Amount of tissue used in luciferase assay	Luminometer reading
Right superficial muscle (M. gracilis)	140 milligram (mg)	0.044
Right deep muscle (M. semimembranosus)	140 mg	0.065
Right Inguinal lymph node	100 mg	0.040
Right Popliteal lymph node	73 mg	0.052
Left superficial muscle	140 mg	0.058
Left Deep muscle	140 mg	13.82
Left Inguinal lymph node	could not locate	Not determined
Left Popliteal lymph node	80 mg	0.078

While no significant luciferase activity was observed in the leg injected with naked DNA, approximately 2 μg total of luciferase was produced in the entire deep muscle of the leg injected with the lipid/DNA formulation (assay sensitivity=2 pg), providing evidence for enhanced gene delivery and antigen production via use of cationic lipid formulations of the present invention.

Example 9. Effect of cationic lipid formulated DNA vaccines in mice.

This example demonstrates that formulation of DNA vaccines with cationic lipids does not enhance nucleic acid efficacy in mice, in contrast to the enhancement of nucleic acid efficacy seen in cats treated with cationic lipid/DNA formulations.

Three different nucleic acid molecules, encoding rabies glycoprotein G, were prepared as described in Example 2. The first, pMV 5044, contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the rabbit beta globin polyadenylation sequence. The second, pMV 5045, contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence. The third, pMV 5046, contains the CMV promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence.

The three nucleic acid molecules encoding rabies glycoprotein G (rabies G) were complexed with LTI lipid 106-3 at a lipid to DNA ratio (μg:μg) of 1:1 as described in Example 2. The corresponding "naked" DNA vaccines were prepared by dissolving the plasmids in saline.

A total of 30 mice were divided into six vaccine groups as follows:

Group 1 (pMV 5044, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 2 (pMV 5044, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 3 (pMV 5045, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 4 (pMV 5045, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 5 (pMV 5046, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 6 (pMV 5046, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Table 5. Anti-rabies G antibody titers of sera samples collected from mice administered a naked DNA vaccine or a complex of the present invention encoding rabies G.

Group 1

mouse #	Formulation	Titer
1	pMV5044, 50 μg + Lipid 106-3	1:40
2	pMV5044, 50 μg + Lipid 106-3	1:40
3	pMV5044, 50 μg + Lipid 106-3	1:51
4	pMV5044, 50 μg + Lipid 106-3	1:115
5	pMV5044, 50 μg + Lipid 106-3	1:135

Group 2

mouse #	Formulation	Titer
1	pMV5044, 100 μg alone	1:43
2	pMV5044, 100 μg alone	1:53
3	pMV5044, 100 μg alone	1:242
4	pMV5044, 100 μg alone	1:1060
5	pMV5044, 100 μg alone	1:3795

Group 3

mouse #	Formulation	Titer
1	pMV5045, 50 μg + Lipid 106-3	1:40
2	pMV5045, 50 μg + Lipid 106-3	1:65
3	pMV5045, 50 μg + Lipid 106-3	1:68
4	pMV5045, 50 μg + Lipid 106-3	1:73
5	pMV5045, 50 μg + Lipid 106-3	1:137

Group 4

mouse #	Formulation	Titer
1	pMV5045, 100 μg alone	1:1
2	pMV5045, 100 μg alone	1:46
3	pMV5045, 100 μg alone	1:100
4	pMV5045, 100 μg alone	1:547
5	pMV5045, 100 μg alone	1:640

Group 5

mouse #	Formulation	Titer
1	pMV5046, 50 μg + Lipid 106-3	1:1
2	pMV5046, 50 μg + Lipid 106-3	1:1
3	pMV5046, 50 μg + Lipid 106-3	1:1
4	pMV5046, 50 μg + Lipid 106-3	1:34
5	pMV5046, 50 μg + Lipid 106-3	1:54

Group 6

mouse #	Formulation	Titer
1	pMV5046, 100 μg alone	1:1
2	pMV5046, 100 μg alone	1:1
3	pMV5046, 100 μg alone	1:1
4	pMV5046, 100 μg alone	1:1
5	pMV5046, 100 μg alone	1:59

Rabies-virus specific neutralizing antibody activity was measured by RFFIT in the sera of all mice four weeks after injection with three different nucleic acid molecules containing Rabies glycoprotein G.

The data presented in Table 5 indicate that cationic lipid formulation of a DNA vaccine does not enhance vaccine efficacy, as measured by humoral (antibody) response, in mice. These data are in contrast to results obtained in cats, where vaccine efficacy is enhanced by formulation with cationic lipids. For the nucleic acid construct pMV5044,

formulation with lipid actually appears to slightly reduce DNA vaccine efficacy for mice, with the geometric means (of the five mice per group) declining from 294 with DNA alone to 66 with DNA/lipid complex. Results from the other two constructs in mice also showed no increase in efficacy; the geometric means were as follows: for pMV5045, 69.4 for DNA alone and 70.7 with DNA/lipid complex; and for pMV5046, 2.3 for DNA alone and 4.5 for DNA/lipid complex.

Example 10. Administration of a DNA plasmid, formulated with and without LTI lipid 106-3, to cats.

This example demonstrates the local immune response at the site of injection of DNA plasmids formulated with or without LTI lipid 106-3. Each of four cats was administered each of the following formulations to each of the following sites on the ventral side: (a) saline (i.e., vehicle alone) to the right arm; (b) 300 μg of lipid 106-3 (lipid alone) to the left arm; (c) 300 μg of a naked plasmid vector encoding rabies glycoprotein G (naked rabies G vector) to the upper right foot; (d) 300 μg of a naked plasmid vector encoding luciferase (naked luciferase vector) to the lower right foot; (e) 300 μg of rabies G vector formulated with 300 μg of lipid 106-3 to the upper left foot; and (f) 300 μg of luciferase vector formulated with 300 μg of lipid 106-3 to the lower left foot.

Six days after administration of the various formulations, the cats were euthanized and muscle and popliteal lymph node muscles were collected. Although the injection sites were marked, it was difficult to obtain muscle samples from the injection sites; thus, only four injection sites were identified, namely those for the saline only and naked rabies G vector in one cat and those for lipid only and rabies G vector plus lipid in another cat. Muscle samples were sectioned using a cryostat and the sections were stained using hematoxylin and eosin to analyze the population of cells infiltrating the sites of injection. Muscle samples were also stained with antibodies specific for B-cells (anti-CD79a antibodies) using techniques known to those skilled in the art.

No differences were seen among the various lymph nodes with respect to cell infiltration. In the muscle samples where vehicle alone, lipid alone or naked rabies G vector was injected, the infiltrating population of cells were mostly macrophage-like cells. In contrast, in the muscle sample where the formulation comprising rabies

G vector complexed with lipid was infected, the infiltrating cells were predominantly lymphocyte-like cells. Staining results with anti-CD79a antibodies suggested that the majority of lymphocytes present were T cells.

These results, as well as others provided herein, suggest that administration of nucleic acid molecules complexed with cationic lipids to cats leads to enhanced expression of the protein encoded by the nucleic acid molecule and infiltration of lymphocytes to the injection site which apparently does not occur when naked nucleic acid molecules are administered in a similar manner. Without being bound by theory, it is believed that this infiltration of lymphocytes might explain the enhanced immune response seen with nucleic acid molecule-cationic lipid complexes of the present invention.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A method to elicit an immune response to an antigen in a felid, said method comprising parenterally administering to said felid a composition comprising a nucleic acid molecule complexed with a cationic lipid, wherein said nucleic acid molecule encodes said antigen.

2. A method to deliver a nucleic acid molecule to a felid, said method comprising parenterally administering a composition comprising said nucleic acid molecule complexed with a cationic lipid.

3. A method to protect a felid from rabies infection, said method comprising parenterally administering to said felid a composition comprising a nucleic acid molecule encoding rabies glycoprotein G, wherein said nucleic acid molecule is complexed with a cationic lipid.

4. The method of Claim 2, wherein said nucleic acid molecule encodes a compound selected from the group consisting of an RNA molecule and a protein.

5. The method of Claim 2, wherein said nucleic acid molecule encodes a protein that elicits an immune response in said felid.

6. The method of Claim 5, wherein said protein is selected from the group consisting of an antigen and an immunomodulator.

7. The method of Claim 1 or 5, wherein said immune response comprises an antibody response.

8. The method of Claim 1 or 5, wherein said immune response comprises a cell-mediated response.

9. The method of Claim 1 or 5, wherein said immune response protects said felid from disease.

10. The method of Claim 1 or 6, wherein said antigen is selected from the group consisting of a protozoan parasite antigen, a helminth parasite antigen, an ectoparasite antigen, a fungal antigen, a bacterial antigen, and a viral antigen.

11. The method of Claim 1 or 6, wherein said antigen is selected from the group consisting of a calicivirus antigen, a coronavirus antigen, a herpesvirus antigen, an immunodeficiency virus antigen, an infectious peritonitis virus antigen, a leukemia virus antigen, a parvovirus antigen, a rabies virus antigen, a *Bartonella* antigen, a *Yersinia*

antigen, a *Dirofilaria* antigen, a *Toxoplasma* antigen, a flea antigen, a flea allergen, a midge antigen, a midge allergen, a mite antigen, a mite allergen, and a tumor antigen.

12. The method of Claim 1 or 6, wherein said antigen comprises rabies glycoprotein G antigen.

13. The method of Claim 1, 2, or 3, wherein said cationic lipid comprises a tetramethyltetraalkyl spermine analog lipid.

14. The method of Claim 1 or 3, wherein said composition further encodes an immunomodulator.

15. The method of Claim 1, 2, or 3, wherein said felid is selected from the group consisting of domestic cats, wild cats, and zoo cats.

16. The method of Claim 1, 2, or 3, wherein the felid is selected from the group consisting of domestic cats, lions, tigers, leopards, panthers, cougars, bobcats, lynx, bobcats, lynx, jaguars, cheetahs, and servals.

17. The method of Claim 1, 2, or 3, wherein the felid is a domestic cat.

18. The method of Claim 1, 3, or 5, wherein a single administration of said composition elicits an immune response.

19. The method of Claim 1, 3, or 6, wherein said step of administering enhances an immune response compared to administration of a naked DNA vaccine encoding said antigen of Claim 1 or 6 or said rabies glycoprotein G of Claim 3 to a felid.

20. The method of Claim 1, 2, or 3, wherein said step of administering is selected from the group of intramuscular administration, intravenous administration, subcutaneous administration, intradermal administration, and intraperitoneal administration.

21. The method of Claim 1, 2, or 3, wherein said step of administering effects about 75% seroconversion in a population of felids administered said nucleic acid molecule.

22. The method of Claim 1, 2, or 3, wherein said step of administering effects about 100% seroconversion in a population of felids administered said nucleic acid molecule.

23. The method of Claim 1, 2, or 3, wherein said nucleic acid molecule:lipid ratio is from about 1:10 to about 10:1.

24. The method of Claim 1, 2, or 3, wherein said nucleic acid molecule is administered in a dose of from about 75 micrograms to about 1,000 micrograms.

25. The method of Claim 1, 2 or 3, wherein said nucleic acid molecule is administered in a dose of not more than about 75 micrograms.

26. The method of Claim 1, 2, or 3, wherein said composition is dehydrated and subsequently rehydrated prior to administration.

27. The method of Claim 1, 2, or 3, wherein said composition further comprises an excipient.

Abstract

The present invention relates to a method to introduce a nucleic acid molecule into a felid by administration of a nucleic acid-cationic lipid complex composition. The method includes the step of administering to the felid, by a parenteral route, a nucleic acid-cationic lipid complex to elicit and/or enhance an immune response. In one embodiment, this method enhances the immune response in a felid compared to a method in which a naked DNA vaccine is administered to a felid. Also provided is a method to deliver a nucleic acid to a felid. This method comprises parenterally administering to the felid a composition that includes a nucleic acid molecule complexed with a cationic lipid.

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(57) Abstract

The present invention relates to a method to introduce a nucleic acid molecule into a felid by administration of a nucleic acid–cationic lipid complex composition. The method includes the step of administering to the felid, by a parenteral route, a nucleic acid–cationic lipid complex to elicit and/or enhance an immune response. In one embodiment, this method enhances the immune response in a felid compared to a method in which a naked DNA vaccine is administered to a felid. Also provided is a method to deliver a nucleic acid to a felid. This method comprises parenterally administering to the felid a composition that includes a nucleic acid molecule complexed with a cationic lipid.

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CATIONIC LIPID-MEDIATED ENHANCEMENT OF NUCLEIC ACID IMMUNIZATION OF CATS

FIELD OF THE INVENTION

The present invention relates to a method to introduce a nucleic acid molecule into a felid by administration of a nucleic acid molecule-cationic lipid complex composition. In particular, the present invention relates to the parenteral administration of a nucleic acid molecule-cationic lipid complex to elicit and/or enhance an immune response to the protein encoded by the administered nucleic acid molecule.

BACKGROUND OF THE INVENTION

Introduction of DNA into an animal for the purpose of eliciting an immune response is often referred to as DNA vaccination. DNA vaccination represents a means of expressing an antigen *in vivo* for the generation of humoral and cellular immune responses. DNA vaccines employ genes encoding antigens, rather than using the proteins themselves, to induce immune responses. The DNA, upon administration to the host, is transcribed and translated *in vivo* to produce an antigen. Processing and presentation of the antigen stimulates the animal's immune system to elicit a humoral and/or cellular response to the antigen. This immune response can potentially confer protective immunity to the animal.

DNA vaccines appear to have advantages over protein antigen-based vaccines, standard "killed" pathogen vaccines, live, attenuated vaccines, and recombinant viral vector vaccines. For example, DNA vaccines appear to be more effective in producing an antigen with a properly folded, native three-dimensional conformation and in generating a cellular immune response than are protein antigens. DNA vaccines also do not exhibit at least some of the safety problems of killed, live or virally-vectored vaccines. For example, a killed virus preparation may contain residual live viruses or may need to be mixed with reactogenic adjuvants, such as those associated with vaccine-related fibrosarcomas in cats, in order to stimulate an effective immune response. An attenuated virus may mutate and revert to a pathogenic phenotype. Viral vector vaccines genetically engineered to express a gene encoding the desired antigen may stimulate the production of antibodies that react with the virus as well; such antibodies may render futile any further attempt to use that virus as a vector, even with a different gene insert.

In contrast, DNA vaccines apparently are non-reactogenic and, if they elicit an immune response, that response is targeted against the antigen of choice.

DNA vaccines typically include a bacterial plasmid, a strong viral promoter, the gene of interest, and a polyadenylation/transcriptional termination sequence. The plasmid is typically grown in bacteria, purified, dissolved in a saline solution, and then simply injected into an animal. Current understanding of how to use DNA vaccines to generate an effective immune response, however, is not complete. Most of our understanding of the mechanisms of DNA vaccine action is derived from rodent studies. In mice, bone marrow-derived antigen-presenting cells have been shown to induce cytotoxic T lymphocyte responses following intramuscular inoculation of naked plasmid DNA. In some cases, DNA vaccination has also been shown to stimulate antigen-specific antibodies, some of which may be neutralizing antibodies. DNA vaccines have also been administered to large animals, albeit with varying degrees of success. While there are some clear examples of DNA vaccine efficacy in large animals, other studies cite relatively weak responses, requirement for large amounts of DNA, or the need for multiple immunizations. As such, it is apparent that further technology development is required to maximize DNA vaccine efficacy in humans and large animals.

Immune responses to DNA vaccination appear to vary according to the vehicle used with the DNA vaccine, the antigen expressed by the DNA vaccine, the route of administration, and the species of mammal into which the DNA vaccine is injected. Investigators have used different vehicles and/or genes encoding cytokines and other stimulatory molecules in an attempt to enhance the immune response to the antigens encoded by DNA vaccines with mixed success. Although cationic lipids have been used to deliver nucleic acids to cells *in vitro* and *in vivo*, there is no consensus in the literature about whether cationic lipids reproducibly enhance the immunogenicity of DNA vaccines. Gregoriadis et al., 1997, FEBS Letters 402, 107, reported that intramuscular (I.M.) injection of DNA encoding HBsA "entrapped" in cationic liposomes into mice elicited an enhanced immune response compared to I.M. injection of "naked" DNA encoding HBsA, whereas DNA encoding HBsA merely "complexed" with cationic lipid generated a reduced immune response compared to "naked" DNA. Ishii et al., 1997, AIDS Research and Human Retroviruses 13, 1421-1424, demonstrated enhanced

immune responses to V3 peptide following I.M., intraperitoneal (I.P.), intradermal (I.D.), intranasal (I.N.) or subcutaneous (S.Q.) administration to mice.

Other investigators, in contrast, found no enhancement of immune responses when cationic lipids were used as a vehicle for DNA vaccines in mice. For example, Davis, et al., 1997, Vaccine 15, 849, found that DNA vaccines encoding the Hepatitis B surface antigen formulated with varying amounts of cationic lipids performed no better than DNA alone in inducing a humoral response in mice. Gramzinski, et al., 1998, Molecular Medicine 4, 109, reported that Aotus monkeys administered DNA vaccines encoding HBsA either with or without cationic lipids (CELLFECTIN®, 10:1 DNA:lipid) by I.M. injection did not seroconvert. Clearly, there is no consensus regarding whether cationic lipids reproducibly act to elicit or enhance immune responses to DNA vaccines.

There also appears to be a high degree of variability of the efficacy of DNA vaccines between different routes of administration. Ishii et al, *ibid.*, for example, found in mice that I.M. and I.N. administration of DNA vaccines generated approximately equivalent immune responses, but that I.P. administration was less effective, and that I.D. and S.Q. administration routes were even less effective. Ishii et al, *ibid.*, found these differences to be consistent regardless of whether DNA was used alone or formulated with cationic lipids. Yokoyama et al, 1996, FEMS Immuno Med Microbio 14, 221-230, showed that I.V. administration of a DNA vaccine generated a better immune response than I.M. administration of the same vaccine in mice.

Taken together, these data indicate that there is a high degree of variability in the effectiveness of DNA vaccines and in the ability of cationic lipids to enhance the effectiveness of DNA vaccines both within and between species and routes of administration.

There are a number of diseases in cats which lead to significant morbidity and mortality. It would be desirable to provide novel and safe vaccines that would confer protective immunity to these diseases. That there is still a need for such vaccines is underscored not only by the association of some feline vaccines with the development of fibrosarcomas but also by the finding that I.M. administration of naked DNA encoding either human growth hormone (hGH) or rabies virus glycoprotein G into domestic cats

resulted in incomplete seroconversion, even after two immunizations (Osorio et al, 1999, Vaccine, in press). These results indicate that parenteral naked DNA vaccination efficacy in cats is inferior to results obtained in mice, and that the efficacy achieved using naked DNA in cats is not sufficient to protect cats from disease. Thus, there remains a need to provide a method to elicit and to enhance the immune response to antigen encoded by DNA vaccines in cats.

SUMMARY OF THE INVENTION

The present invention relates to a method to elicit an immune response to an antigen in a felid. This method includes the step of parenterally administering to the felid a composition comprising a nucleic acid molecule encoding the antigen in which the nucleic acid molecule is complexed with a cationic lipid. In one embodiment, this method enhances the immune response in a felid compared to a method in which a naked DNA vaccine is administered to a felid. Also provided is a method to deliver a nucleic acid molecule to a felid. This method comprises parenterally administering to the felid a composition that includes a nucleic acid molecule complexed with a cationic lipid.

DETAILED DESCRIPTION

The present invention relates to a method to elicit an immune response to an antigen in a felid. The method includes the step of parenterally administering to the felid a composition comprising a nucleic acid molecule encoding the antigen in which the nucleic acid molecule is complexed with a cationic lipid. The ability of such a method to elicit an immune response to the antigen encoded by the nucleic acid molecule is new and surprising. Until recently, the general perception of those skilled in the art was that cationic lipids did not enhance the ability of a nucleic acid molecule to elicit an immune response, compared to, for example, delivery of a naked, or unformulated, nucleic acid molecule (i.e., a nucleic acid molecule that is not complexed with, for example, a lipid or other transfection-facilitating agents). Recent studies, cited above, have provided conflicting results: although two studies in mice demonstrated that cationic lipids enhanced the ability of DNA to elicit an immune response, a third study concluded that cationic lipid-complexed DNA was no better than naked DNA at eliciting an immune response. In addition, monkeys administered a nucleic acid molecule-cationic lipid

complex did not exhibit seroconversion to the antigen encoded by the nucleic acid molecule. Furthermore, the inventors have demonstrated that while parenteral administration to a felid of a nucleic acid molecule complexed with a cationic lipid results in the felid successfully seroconverting in response to the antigen encoded by the nucleic acid molecule, intranasal administration of such a composition did not result in seroconversion. Thus, the ability to demonstrate seroconversion in cats parenterally administered a nucleic acid molecule complexed with a cationic lipid is completely unpredictable based on previous studies and, as such, is inventive.

One embodiment of the present invention is the use of a composition comprising a nucleic acid molecule encoding an antigen complexed with a cationic lipid to elicit an immune response in a felid. It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, a nucleic acid molecule, an antigen, and a cationic lipid refers to one or more nucleic acid molecules, antigens, and cationic lipids, respectively; or to at least one nucleic acid molecule, antigen, and cationic lipid, respectively. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, a member of a group that is "selected from the group consisting of" refers to one or more of members of that group, including combinations thereof.

A nucleic acid molecule of the present invention also referred to herein as a nucleic acid, can be DNA or RNA. In one embodiment, a nucleic acid molecule encodes an antigen that elicits an immune response in a felid. As such, a nucleic acid molecule can simply be a molecule that encodes such an antigen, i.e., a coding region, or the nucleic acid molecule can comprise a coding region operatively linked to a regulatory sequence. As used herein, the phrase operatively linked refers to the joining of a coding region to one or more regulatory sequences such that the coding region is expressed using such regulatory sequence(s) in a felid. Examples of such regulatory sequences include transcription control sequences and translation control sequences that can be recognized by felid cellular mechanisms in order to effect transcription and translation of a coding region. Transcription control sequences are sequences that control the initiation, elongation, and termination of transcription (e.g., promoters,

enhancers, introns, polyA sites, terminators). Translation control sequences control the initiation, elongation and termination of translation. Additional regulatory sequences include signal sequences that effect secretion of a protein from a cell and a combination of a signal sequence and a transmembrane sequence (i.e., membrane anchoring domain) that causes a protein to be partially extracellular and partially retained in the membrane and/or cytoplasm. A preferred nucleic acid molecule of the present invention is a plasmid or viral genome that includes a coding region for the desired antigen operatively linked to strong eukaryotic regulatory sequences, including a strong promoter and strong transcription termination/polyadenylation sequences. A preferred plasmid can replicate in bacteria. Procedures by which such a nucleic acid molecule is produced are known to those skilled in the art, and are disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press. Appropriate plasmids are known in the art, and may include, but are not limited to, pUC19 and BLUESCRIPT®. A preferred plasmid is pUC19. Appropriate regulatory sequences are known to those skilled in the art. For example, a suitable promoter includes, but is not limited to the cytomegalovirus immediate early promoter (CMV IE) with or without intron A, a long terminal repeat (LTR) promoter from a retrovirus, or a strong cellular promoter such as β-actin, with CMV IE with intron A being preferred. Similarly, suitable transcription termination sequences include, but are not limited to, bovine growth hormone, SV40 virus or rabbit beta-globin polyadenylation sequences, with a bovine growth hormone sequence being preferred.

A suitable antigen is any antigen that effects an immune response, and as such includes allergens and autoantigens as well as other antigens. An antigen, as used herein, can refer to the full-length antigen or any portion thereof that is capable of eliciting an immune response. Preferred antigens are those that elicit an immune response that protects an animal from disease. Examples of such antigens include, but are not limited to, a protozoan parasite antigen, a helminth parasite antigen, an ectoparasite antigen, a fungal antigen, a bacterial antigen, and a viral antigen. Examples of viral antigens include, but are not limited to, antigens from adenoviruses, caliciviruses, coronaviruses, distemper viruses, hepatitis viruses, herpesviruses, immunodeficiency viruses, infectious peritonitis viruses, leukemia viruses, oncogenic

viruses, papilloma viruses, parainfluenza viruses, parvoviruses, rabies viruses, and reoviruses, as well as other cancer-causing or cancer-related viruses. Examples of bacterial antigens include, but are not limited to, antigens from *Actinomyces, Bacillus, Bacteroides, Bordetella, Bartonella, Borrelia, Brucella, Campylobacter, Capnocytophaga, Clostridium, Corynebacterium, Coxiella, Dermatophilus, Enterococcus, Ehrlichia, Escherichia, Francisella, Fusobacterium, Haemobartonella, Helicobacter, Klebsiella*, L-form bacteria, *Leptospira, Listeria, Mycobacteria, Mycoplasma, Neorickettsia, Nocardia, Pasteurella, Peptococcus, Peptostreptococcus, Proteus, Pseudomonas, Rickettsia, Rochalimaea, Salmonella, Shigella, Staphylococcus, Streptococcus*, and *Yersinia*. Examples of fungal antigens include, but are not limited to, antigens from *Absidia, Acremonium, Alternaria, Aspergillus, Basidiobolus, Bipolaris, Blastomyces, Candida, Chlamydia, Coccidioides, Conidiobolus, Cryptococcus, Curvalaria, Epidermophyton, Exophiala, Geotrichum, Histoplasma, Madurella, Malassezia, Microsporum, Moniliella, Mortierella, Mucor, Paecilomyces, Penicillium, Phialemonium, Phialophora, Prototheca, Pseudallescheria, Pseudomicrodochium, Pythium, Rhinosporidium, Rhizopus, Scolecobasidium, Sporothrix, Stemphylium, Trichophyton, Trichosporon*, and *Xylohypha*. Example of protozoan and helminth parasite antigens include, but are not limited to, antigens from *Babesia, Balantidium, Besnoitia, Cryptosporidium, Eimeria, Encephalitozoon, Entamoeba, Giardia, Hammondia, Hepatozoon, Isospora, Leishmania*, Microsporidia, *Neospora, Nosema, Pentatrichomonas, Plasmodium, Pneumocystis, Sarcocystis, Schistosoma, Theileria, Toxoplasma*, and *Trypanosoma, Acanthocheilonema, Aelurostrongylus, Ancylostoma, Angiostrongylus, Ascaris, Brugia, Bunostomum, Capillaria, Chabertia, Cooperia, Crenosoma, Dictyocaulus, Dioctophyme, Dipetalonema, Diphyllobothrium, Diplydium, Dirofilaria, Dracunculus, Enterobius, Filaroides, Haemonchus, Lagochilascaris, Loa, Mansonella, Muellerius, Nanophyetus, Necator, Nematodirus, Oesophagostomum, Onchocerca, Opisthorchis, Ostertagia, Parafilaria, Paragonimus, Parascaris, Physaloptera, Protostrongylus, Setaria, Spirocerca, Spirometra, Stephanofilaria, Strongyloides, Strongylus, Thelazia, Toxascaris, Toxocara, Trichinella, Trichostrongylus, Trichuris. Uncinaria*, and *Wuchereria*. Examples of ectoparasite antigens include, but are not limited to, antigens

(including protective antigens as well as allergens) from fleas; ticks, including hard ticks and soft ticks; flies, such as midges, mosquitos, sand flies, black flies, horse flies, horn flies, deer flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats; ants; spiders, lice; mites; and true bugs, such as bed bugs and kissing bugs. Additional examples of suitable allergens include food, grass, weed, tree pollen, other animal and other plant allergens.

Preferred antigens include, but are not limited to, a calicivirus antigen, a coronavirus antigen, a herpesvirus antigen, an immunodeficiency virus antigen, an infectious peritonitis virus antigen, a leukemia virus antigen, a panleukopenia virus antigen, a parvovirus antigen, a rabies virus antigen, a *Bartonella* antigen, a *Yersinia* antigen, a *Dirofilaria* antigen, a *Toxoplasma* antigen, a tumor antigen, a flea antigen, a flea allergen, a midge antigen, a midge allergen, a mite antigen, a mite allergen, a ragweed allergen, a ryegrass allergen, a cat allergen, a dog allergen, a Bermuda grass allergen, a Johnson grass allergen, or a Japanese cedar pollen allergen. Particularly preferred antigens include a rabies virus glycoprotein G antigen; heartworm PLA2, P39, P4, P22U, Gp29, astacin, cysteine protease, macrophage migration inhibitory factor, venom allergen, TPX-1, TPX-2, transglutaminase, ankyrin, asparaginase, calreticulin, cuticulin, and aromatic amino aid decarboxylase antigens; flea serine protease, cysteine protease, aminopeptidase, serpin, carboxylesterase, juvenile hormone esterase, chitinase, epoxide hydrolase, ecdysone, ecdysone receptor, and ultraspiracle protein antigens; flea salivary antigens; *Yersinia* F1 and V antigens; and *Toxoplasma gondii* antigens such as those disclosed in PCT Patent Publication No. WO 99/32633, published July 1, 1999, by Milhausen et al. Additional examples of suitable and preferred allergens are disclosed in U.S. Patent No. 5,945,294, issued August 31, 1999, by Frank et al. (US 5,945,294); U.S. Patent No. 5,958,880, issued September 28, 1999, by Frank et al. (US 5,958,880); PCT Patent Publication No. WO 98/45707, published October 15, 1998, by Frank et al. (WO 98/45707); and PCT Patent Publication No. WO 99/38974, published August 5, 1999, by Weber et al. (WO 99/38974).

One embodiment of the present invention is a composition comprising a nucleic acid molecule-cationic lipid complex that further comprises a heterologous nucleic acid molecule encoding an immunomodulator. Such an immunomodulator-encoding nucleic

acid molecule can be contained within the same nucleic acid molecule encoding the antigen of the present invention, or can exist as a separate nucleic acid molecule, which can be on the same or separate plasmid or viral genome. The present invention also includes Suitable immunomodulators include compounds that enhance certain immune responses as well as compounds that suppress certain immune responses. Compounds that enhance the immune response include compounds that preferentially enhance humoral immunity as well as compounds that preferentially enhance cell-mediated immunity. Suitable compounds can be selected depending on the desired outcome. Suitable immunomodulators include, but are not limited to, cytokines, chemokines, superantigens, co-stimulatory molecules, adhesion molecules, and other immunomodulators as well as compounds that induce the production of such immunomodulators. Examples of such compounds include, but are not limited to, granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), erythropoietin (EPO), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 18 (IL-18), interferon gamma, interferon gamma inducing factor I (IGIF), transforming growth factor beta (TGF-β), RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory proteins (e.g., MIP-1 alpha and MIP-1 beta), Leishmania elongation initiating factor (LEIF), B7-1, B7-2, CD40, CD40 ligand, ICAM-1, and VCAM.

A composition of the present invention includes a cationic lipid complexed with a nucleic acid molecule encoding an antigen in order to elicit or enhance an immune response to the antigen. As used herein, a cationic lipid is a lipid which has a cationic, or positive, charge at physiologic pH. Cationic lipids can have a variety of forms, including liposomes or micelles. Whether a cationic lipid occurs primarily as a liposome or a micelle can be manipulated by methods known in the art; for example, a freezing and thawing of cationic lipids in aqueous solution will encourage formation of liposomes, rather than micelles. A nucleic acid molecule complexed with a cationic lipid may also be referred to as a nucleic acid molecule-cationic lipid complex, a

lipoplex or a complex of the present invention. A complex of the present invention that elicits an immune response is a complex of a nucleic acid molecule which encodes an antigen with a cationic lipid. As used herein, the term complexed with, which is equivalent to complexed to, refers to any method by which a nucleic acid molecule interacts (e.g. binds, comes into contact with a cationic lipid.) Such an interaction can include, but is not limited to encapsulation of a nucleic acid molecule into a cationic liposome, association of a nucleic acid molecule and cationic lipid characterized by non-covalent, ionic charge interactions, and other types of associations between nucleic acid molecules and cationic lipids known by those skilled in the art. It is preferred that cationic lipids have a cationic group, such as a quaternary amine group, and one or more lipophilic groups, such as saturated or unsaturated alkyl groups having from about 6 to 30 carbon atoms. Cationic lipid compositions suitable for use in the present invention include lipid compositions comprised of one type of lipid, or lipid compositions comprised of more than one type of lipid. If there is more than one type of lipid present in a lipid composition, it is necessary that the overall net charge of the lipid composition is cationic, i.e. positive; however, as long as the overall net charge of the lipid composition is cationic, individual lipid types may be neutral or even anionic in charge. A composition of the present invention includes a cationic lipid that is suitable in accordance with the present invention. Cationic lipids suitable for use in the present invention include commercially available cationic lipids, for example DOTMA, available under the trademark name of LIPOFECTIN®, available from Life Technologies Inc., (LTI), Gaithersburg, MD and DDAB, available from Boehringer-Mannheim, Indianapolis, IN. In addition, suitable cationic lipids can be synthesized as described in the literature; see, for example, Felgner et al., 1987, PNAS 84 7413-7417 regarding the preparation of DOTAP; Douar et al, 1996, Gene Ther 3(9), 789-796 regarding the preparation of Lipid 67; Wheeler et al., 1996, Biochim Biophys Acta 1280(1), 1-11 regarding the preparation of DMRIE; McLean et al., 1997 Am J Physiol 273, H387-404 regarding the preparation of DOTIM; and Hofland et al., 1997, Pharm Res 14(6), 742-749 regarding the preparation of DOSPA. Other suitable cationic lipid compounds are described in the literature. See, for example, Stamatatos et al., 1988, Biochemistry 27, 3917-3925 and Eibl, et al., 1979, Biophysical Chemistry 10, 261-271.

Preferred cationic lipids include the class of lipids known as tetramethyltetraalkyl spermine analogs, described by McCluskey et al., (1998), *Antisense and Nucleic Acid Drug Development*, vol. 8, pp 401-414. Lipids of this type include tetramethyltetralaurylspermine, tetramethyltetramyristylspermine, tetramethyltetrapalmitoylspermine, and tetramethyltetraoleoylspermine. The following lipids, obtained from LTI are of the tetramethyltetraalkyl spermine class, with the alkyl groups containing fatty acid chains of length longer than oleic acid. These lipids are denoted as LTI lipids 4251-781-1, 4251-106-3, 4518-52, D304-200, 4521-52-3, 4251-106-4, 4251-781-2, 4518-53, 4518-31, 4519-30, 4519-34, and 2518-111. Preferred cationic lipids include LTI lipid 4251-781-1, LTI lipid 4251-106-3, and LTI lipid 4518-52. In one embodiment, tetramethyltetraalkyl spermine lipids are formulated with a neutral lipid, such as dioleylphosphatidyl-ethanolamine (DOPE).

A nucleic acid molecule-cationic lipid complex can be formed by using techniques known to those skilled in the art, examples of which are described in the Examples section. A complex can be formed, for example, by adding a cationic lipid solution to a nucleic acid molecule, preferably an endotoxin-free nucleic acid molecule, at concentrations appropriate for the present invention, and mixing, for example by pipetting. Preferable nucleic acid molecule-to-cationic lipid ratios are from about 10:1 weight nucleic acid molecule: weight cationic lipid, (e.g. microgram (μg) nucleic acid molecule to μg cationic lipid) to about 1:10 weight nucleic acid molecule: weight cationic lipid. More preferable are ratios from about 1:2 weight of nucleic acid molecule: cationic lipid to about 4:1 weight of nucleic acid molecule: cationic lipid. In a preferred embodiment, the nucleic acid molecule-cationic lipid complex is incubated at room temperature for about 30 minutes before administration. A nucleic acid molecule-cationic lipid complex can be dehydrated and rehydrated using techniques known to those skilled in the art; for example, the complex can be frozen in liquid nitrogen and lyophilized at 150 milliTorr, then reconstituted in solution for injection.

A dose of a nucleic acid molecule-cationic lipid complex to administer to a cat can be reported as the amount of nucleic acid molecule administered to a cat. A preferred dose of a nucleic acid molecule-cationic lipid complex to administer to a cat includes from at least one nanogram (ng) of nucleic acid to about 10 milligram (mg) of

nucleic acid molecule. More preferred is a dose range that includes from about 1 μg nucleic acid molecule to about 1 mg of nucleic acid molecule. Particularly preferred is a dose ranging from about 75 μg of a nucleic acid molecule to about 300 μg of a nucleic acid molecule.

A nucleic acid molecule-cationic lipid complex composition of the present invention can be formulated in an excipient that the animal to be treated can tolerate. As such, the present invention includes administration of a composition comprising a nucleic acid molecule-cationic lipid complex, wherein the composition further comprises an excipient. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, mannitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

In one embodiment of the present invention, the nucleic acid molecule-cationic lipid complex can also include an adjuvant and/or a carrier. One advantage of a nucleic acid molecule-cationic lipid complex is that adjuvants and carriers are not required to produce a composition that administration thereof will elicit an immune response. However, it should be noted that use of adjuvants or carriers is not precluded by the present invention. Adjuvants are typically substances that generally enhance the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, other bacterial cell wall components; aluminum-based salts; calcium-based salts; silica; polynucleotides; toxins, such as cholera toxin; toxoids, such as cholera toxoid; serum proteins; other viral coat proteins; other bacterial-derived preparations; block copolymer adjuvants, such as Hunter's Titermax™ adjuvant (Vaxcel™, Inc. Norcross, GA); Ribi adjuvants (available from Ribi ImmunoChem

Research, Inc., Hamilton, MT); and saponins and their derivatives, such as Quil A (available from Superfos Biosector A/S, Denmark). Carriers are typically compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release formulations, biodegradable implants, liposomes, bacteria, viruses, oils, esters, and glycols.

An immune response to an antigen includes a humoral, i.e. antibody, response to that antigen and/or a cell-mediated response to that antigen. Methods to measure an immune response are known to those skilled in the art; examples of such methods are disclosed herein. If one or both types of immune response are present, they may protect the felid from disease caused, for example, by the agent from which the antigen was derived. In accordance with the present invention, the ability of an antigen derived from a disease-causing agent to protect an animal from a disease caused by that disease-causing agent or a cross-reactive agent refers to the ability of a nucleic acid molecule-cationic lipid complex of the present invention to treat, ameliorate and/or prevent disease caused by the disease-causing agent or cross-reactive agent, preferably by eliciting an immune response against the antigen derived from the disease-causing agent. It is to be noted that an animal may be protected by a composition of the present invention even without the detection of a humoral or cell-mediated response to the antigen. Protection can be measured by methods known to those skilled in the art, such as by challenging an animal with the agent against which the animal has mounted a putative immune response. In certain cases, the antibody titer of an animal can be used to demonstrate protection. For example, it is known that animals that elicit an antibody response against a rabies glycoprotein G antigen are protected if their sera exhibits a rapid focus fluorescent inhibition test (RFFIT) titer of rabies virus neutralizing antibodies of greater than 1:5. As used herein, an animal that elicits an immune response to an antigen is an animal that has been immunized with that antigen.

The biological mechanism for eliciting and/or enhancing an immune response by the use of a nucleic acid molecule-cationic lipid complex composition of the present invention has not been elucidated, but, without being bound by theory, the inventors believe that the mechanism is likely related to the ability of these compositions to protect DNA from nuclease attack, to facilitate the transfection of both muscle cells and

professional antigen presenting cells (APC) *in vivo*, to increase levels of expression in transfected cells, and/or to distribute DNA to lymphoid organs.

A felid, as used herein, is a member of the family Felidae. Examples of felids include domestic cats, wild cats, and zoo cats. Examples of cats, include, but are not limited to, domestic cats, lions, tigers, leopards, panthers, cougars, bobcats, lynx, jaguars, cheetahs, and servals. A preferred cat to immunize is a domestic cat. The term cat(s) and felid(s) are used interchangeably herein.

As used herein, parenteral administration means administration not through the alimentary canal (e.g. oral administration), but rather by injection through some other route, including but not limited to routes such as subcutaneous, intramuscular (I.M.), intravenous (I.V.), intraperitoneal (I.P.), intradermal (I.D.), intraorbital, intracapsular, intraspinal, and intrasternal. Parenteral administration includes, but is not limited to, administration by any route that includes use of a needle to insert material into the body. Parenteral administration also includes uses of devices other than a syringe and needle to insert material through the skin and or mucosal surfaces into the body, including but not limited to the BIOJECTOR®, POWDERJECT, and MEDIJECT® needleless injection systems. A preferred route of administration includes intramuscular administration using a needle and syringe.

Acceptable protocols to administer therapeutic compositions in an effective manner include individual dose size, number of doses, and frequency of dose administration. Typically, the first administration of a composition intended to elicit an immune response is called the primary (or prime) administration, also known as the pre-boost. Additional administrations intended to "boost" or increase an immune response to an antigen are termed booster administrations. Determination of a protocol to elicit an immune response in a cat using a nucleic acid molecule-cationic lipid complex of the present invention can be accomplished by those skilled in the art. In one embodiment of the present invention, a nucleic acid molecule encoding a desired antigen complexed with cationic lipid need only be administered once by a route appropriate to the present invention (e.g. parenteral) to stimulate an immune response against the antigen. In a preferred embodiment, such an administration protects the felid from the agent from

which the antigen was derived or from an agent against which the immune response is cross-protective.

In one embodiment, administration of a complex of the present invention to a felid in order to elicit an immune response actually enhances the immune response generated by the felid as compared to the immune response generated upon administration of a naked DNA vaccine to a felid, wherein the naked DNA vaccine consists essentially of a naked DNA molecule; i.e., a DNA molecule that is not complexed with lipids. Finding that a complex of the present invention enhances an immune response is surprising both in view of the conflicting studies known to those skilled in the art as described herein as well as in view of the studies described in more detail in the Examples, in which administration of naked DNA vaccines to cats elicited immune responses in only some cats within each group, or population, tested, whereas administration of a complex of the present invention could result in up to 100% seroconversion of all cats in a population tested. As used herein, enhancement of the immune response can include increasing the amount, or titer, of antibody elicited by a complex of the present invention that encodes an antigen to the desired antigen and/or agent from which the antigen was derived as compared to the titer of antibody generated by a naked DNA vaccine that encodes the same antigen. In one embodiment, such an enhancement can be induction of no antibody titer with a naked DNA vaccine to induction of a protective antibody titer with a complex of the present invention. Enhancement of an immune response can also refer to augmentation of the cell-mediated response to the antigen and/or agent encoded by a complex of the present invention as compared to the response generated by a naked DNA vaccine encoding the same antigen. Enhancement of immune response can also include conferring or augmenting protection from disease by a complex of the present invention compared to the protection, if any, conferred by a naked DNA vaccine encoding the same antigen. In one embodiment, enhancement of the immune response includes increasing the likelihood of a cat seroconverting in response to antigen encoded by a complex of the present invention in comparison to the likelihood of the cat responding to the same antigen encoded by a naked DNA vaccine. In other words, in a group of cats being vaccinated with a complex of the present invention, a greater number of cats will

seroconvert in response to antigen encoded by the complex rather than to the same antigen encoded by a naked DNA vaccine. Preferably, the likelihood that a cat will seroconvert when administered a single dose of a complex of the present invention that encodes an antigen is at least about 50%, preferably at least about 75%, more preferably at least about 90% and even more preferably at least about 100%. In the case where a primary and booster administration of the complex is administered, the likelihood that a cat will seroconvert is preferably at least about 75%, more preferably at least about 90%, and even more preferably at least about 100%.

The present invention includes a method to administer a nucleic acid molecule to a felid. The method includes the step of parenterally administering a composition comprising said nucleic acid molecule complexed with a cationic lipid. Such a nucleic acid molecule can encode either a protein or a RNA molecule. In one embodiment, the nucleic acid molecule encodes a protein or RNA molecule that, when expressed at an appropriate level, has a protective effect upon the cat. As used herein, a protein refers to a full-length protein or any portion thereof that is at least about 5 amino acids in length and has a useful function, including, but not limited to, ability to elicit an immune response, elicit an immunomodulatory effect (e.g., an immunomodulator that stimulates or reduces the immune response), effect gene therapy, effect enzyme activity, or otherwise effect cell division, differentiation, development and cell death. As used herein, a RNA molecule refers to any RNA species that can be encoded by a nucleic acid molecule, including, but not limited to antisense RNA, a molecule capable of triple helix formation, a ribozyme, or other nucleic acid-based drug compound. As such, any protein or RNA molecule that can be expressed at an appropriate level in a cat, which protects a cat from disease, would be included in this invention. Diseases from which to protect a felid include, but are not limited to, infectious diseases, genetic diseases, oncological diseases, and other metabolic diseases, including diseases that lead to abnormal cell growth, degenerative processes, and immunological defects. Compositions of the present invention can protect animals from a variety of diseases including, but not limited to, allergies, arthritic diseases, autoimmune diseases, cancers, cardiovascular diseases, graft rejection, hematopoietic disorders, immunodeficiency diseases, immunoproliferative diseases, immunosuppressive disorders, infectious

diseases, inflammatory diseases, jaundice, septic shock, and other immunological defects, as well as other genetic or metabolic defects. Methods to produce and use a composition comprising any nucleic acid molecule of the present invention complexed with any cationic lipid of the present invention are as described herein.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

EXAMPLES

Example 1

This Example demonstrates the production of a nucleic acid molecule of the present invention.

A nucleic acid molecule encoding human growth hormone (hGH) was constructed using plasmid pHGH107 (available from American Type Culture Collection, Manassis, VA), which encodes hGH amino-acids 1-191, as a polymerase chain reaction (PCR) template. The hGH open reading frame was amplified by PCR using Pfu DNA polymerase (available from Stratagene, La Jolla, CA) and the following forward and reverse primers: 5' TTCCCAACTATACCACTATCTCGTCTA 3' (SEQ ID NO:1) and 5' CTAGAAGCCACAGCTGCCCTCCACAGAG 3' (SEQ ID NO:2). The PCR product containing the sequence encoding the mature hGH product was ligated into the *Nae*I site of a plasmid containing the human cytomegalovirus immediate early promoter, a translation control sequence, a sequence encoding the signal peptide coding sequence from human tissue plasminogen activator, and a bovine growth hormone poly A sequence. The expression of hGH from this plasmid was confirmed following transfection of cells *in vitro* and was detected using a chemiluminescence assay kit (available from Nichols Institute Diagnostics, San Juan Capistrano, CA).

A nucleic acid molecule encoding the rabies virus glycoprotein G was described previously and contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence. See Osorio, et al. (1999) Vaccine, in press.

Example 2

This Example describes the production of a nucleic acid molecule-cationic lipid complex of the present invention.

Endotoxin-free nucleic acid molecules encoding hGH or rabies glycoprotein G were prepared using a commercial kit (Qiagen, Inc., Valencia, CA) and the resulting nucleic acid molecules were dissolved in endotoxin-free 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 2 mg per milliliter (ml) to form a hGH nucleic acid molecule solution and a rabies gG nucleic acid molecule solution, respectively. Cationic lipids 4251-106-3 (also known as 106-3), 4251-781-1 (also known as 781-1), and 4518-52 were obtained from Life Technologies, Inc. (LTI), Gaithersburg, MD. A nucleic acid molecule-cationic lipid complex was formed by adding 250 μl of the respective cationic lipid solution to 250 μl of the respective nucleic acid molecule solution, followed by immediate mixing by pipetting. The concentrations of the cationic lipid solutions and of the nucleic acid molecule solutions used were adjusted to give the desired amounts and ratios of nucleic acid molecules to cationic lipids described elsewhere in the Examples. The mixture was incubated at room temperature for 30 minutes before administration. For dehydration and rehydration of a nucleic acid molecule-cationic lipid complex, the complex was frozen in liquid nitrogen and lyophilized at 150 mTorr, then reconstituted in the original volume of sterile water for injection.

Example 3

This Example describes a method for administering a nucleic acid molecule-cationic lipid complex of the present invention to a felid.

Primary and booster administrations of nucleic acid molecule-cationic lipid complexes prepared as described in Example 2 were injected intramuscularly into the semitendinosus or semimembranosus muscle of domestic cats. Each dose was divided into two equal portions and administered bilaterally into each leg. Sera samples were collected every 10 days for antibody determination.

Example 4

This Example describes methods to measure immune responses generated in response to the administration of nucleic acid molecule-cationic lipid complexes of the present invention.

Antibody responses specific for hGH were determined by ELISA. Briefly, ELISA plate wells were coated with 0.4 micrograms (μg) hGH protein per well (hGH protein available from Genzyme Diagnostics, San Carlos, CA) and incubated overnight

at 4°C. Unbound antigen was aspirated and the plate was blocked with 2% skimmed milk for 1 hour at 37°C . ELISA plates were washed 3 times with TBS-Tween (150 milliMolar (mM) NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% TWEEN-20) and serially diluted sera samples from vaccinated cats were added and incubated at 37°C for 1 hour. Plates were washed 3 times with TBS-Tween. A biotin conjugated monoclonal anti-cat IgG (1:30,000) (available from Sigma-Aldrich, St. Louis, MO), was added and incubated for 1 hour at 37°C, followed by the addition of EXTRAVIDIN®-horseradish peroxidase diluted 1:1000, available from Sigma-Aldrich, St. Louis, MO. After a final incubation at 37°C, for 1 hour, the plates were washed and an o-phenylenediamine dihydrochloride substrate solution, available from Sigma-Aldrich, was added and the plates incubated at room temperature for 30 minutes for color development. The plates were read at 450 nm.

Rabies virus-specific neutralizing antibody response were determined using the Rapid Fluorescent Focus Inhibition test (RFFIT) at the Department of Veterinary Diagnostics, Kansas State University.

T cell proliferation assays were carried out in the following manner. Heparinized blood samples were collected from cats a week after administration of a booster injection as described in Example 5. The lymphocytes were isolated from the blood samples using a percoll gradient (Sigma Chemicals, St Louis, MO). The isolated lymphocytes were resuspended in RPMI 1640 (Sigma Chemical) containing 5% normal cat serum, 2 mM L-glutamine (Life Technologies, Bethesda, MD), 1 mM sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Life Technologies), 5 μg/mL gentamycin (Sigma Chemical), 0.1 mM MEM non-essential amino acids (Life Technologies), and 1% essential amino acids (Life Technologies) plated at a density of 2×10^5 cells/well and treated with various concentrations of recombinant human growth hormone (hGH) (Genzyme Diagnostics, Boston, MA) for a total of 3 or 5 days. Each group of cell samples contained a negative control (media alone) and a positive control (Concanavalin A, Sigma Chemicals). Cells were pulsed at time of measurement with 0.5 μCurie of tritiated thymidine (ICN Pharmaceuticals) per well. T cells that were specific for hGH proliferated in response to added hGH and incorporated the tritiated thymidine into their DNA. The amount of incorporated tritium was counted 16 to 18

hours post-pulse in a scintillation counter. Data was reported as the stimulation index, which was derived by dividing the counts per minute obtained from the samples divided by the counts per minute obtained from the negative control.

Example 5

This Example compares the immune response elicited using a nucleic acid molecule encoding hGH complexed with either LTI lipid 781-1 or LTI lipid 106-3 to the immune response elicited using a naked DNA vaccine encoding hGH in cats.

The hGH nucleic acid molecule was complexed with LTI lipid 781-1 at a lipid-to-DNA ratio (μg:μg) of 0.5:1.0, and formulated with LTI lipid 106-3 at a lipid-to-DNA ratio of 1:1, as described in Example 2. The naked DNA vaccine consisted of the hGH nucleic acid molecule prepared as described in Example 2 dissolved in saline.

A total of 12 cats were divided into three vaccine groups as follows:

Group 1 (naked DNA): Two injections, spaced 8 weeks apart, of 300 μg of naked hGH nucleic acid molecule in 500 μl saline.

Group 2 (LTI lipid 781-1): Two injections, spaced 8 weeks apart, of 300 μg hGH nucleic acid molecule complexed with 150 μg cationic lipid.

Group 3 (LTI lipid 106-3): Two injections, spaced 8 weeks apart, of 300 μg hGH nucleic acid molecule complexed with 300 μg cationic lipid.

At day 54 post injection, the cats were boosted with another injection of the appropriate cationic lipid-DNA mixture. At day 111, cats were boosted again, and at day 119, T-cell proliferation assays were performed as described. A T-cell stimulation index of 2 is taken as the cutoff and values below 2 are considered non-responsive.

Sera samples were collected from cats following the primary and booster administrations of complex as described in Example 3 and were assayed for hGH specific antibody responses by ELISA. Endpoint ELISA titers are shown in Table 1. The lowest sera titers measured were 1:40. Therefore, negative titers are expressed as <1:40.

Table 1. hGH antibody titers of sera samples collected from cats administered a naked DNA vaccine or a complex of the present invention encoding hGH

cat #	Formulation	Titer at day 54 (post prime)	Titer at day 64 (post boost)	T-cell stimulation index
1	Naked DNA	<1:40	<1:40	9.1
2	Naked DNA	<1:40	<1:40	2.4
3	Naked DNA	<1:40	1:1125	12.7
4	Naked DNA	<1:40	<1:40	11.3
		geometric mean=40	geometric mean=92	
5	DNA + lipid 781-1	<1:40	<1:40	1.6
6	DNA + lipid 781-1	1:160	1:10,240	20.2
7	DNA + lipid 781-1	1:2312	1:7762	3.7
8	DNA + lipid 781-1	1:233	1:21,183	8.9
		geometric mean=242	geometric mean=2865	
9	DNA + lipid 106-3	1:1076	1:19,000	24.3
10	DNA + lipid 106-3	1:316	1:19,135	65.5
11	DNA + lipid 106-3	<1:40	<1:40	18.2
12	DNA + lipid 106-3	1:125	1:8693	2.6
		geometric mean=203	geometric mean=3353	

The results in Table 1 indicate that there was no seroconversion in any of the four cats administered a single inoculation of 300 μg of the naked hGH nucleic acid molecule. Moreover, following the booster administration, only one of the four cats in the naked DNA vaccine group developed an hGH-specific antibody response. In contrast to the naked DNA vaccine group, 75% of the cats (i.e., 3 of 4 cats) cats in each of the two lipid groups developed detectable titers following the primary administration of complex, and these responses went up markedly following the booster administration of complex.

T-cell proliferation, measured by the T cell stimulation index, indicates that all treatments, including treatment with DNA alone, appeared to activate cell-mediated immunity. Treatment with a complex of DNA and lipid 106-3 appears to work better for

stimulating T cell proliferation in cats than did naked DNA alone or DNA complexed with lipid 781-1.

Example 6

This Example compares immune responses elicited using a nucleic acid molecule encoding rabies glycoprotein G complexed with several cationic lipids of the present invention to the immune response elicited using a naked DNA vaccine encoding rabies glycoprotein G in cats.

This example compared the abilities of the following compositions to elicit an immune response against rabies glycoprotein G (rabies G) in cats: a naked DNA vaccine consisting of the rabies G nucleic acid molecule; and complexes between the rabies G nucleic acid molecule and one of the following cationic lipids: LTI lipid 106-3, LTI lipid 781-1, or LTI lipid 4518-52, each at a variety of DNA:lipid ratios. Also tested was a complex that had been dehydrated by lyophilization and rehydrated prior to administration. Each of the compositions was produced as described in Example 2. All cats received two intramuscular injections as described in Example 3, spaced four weeks apart. The following groups of 4 cats each were tested:

Group 1: Naked DNA, 300 μg rabies G vector

Group 2: 300 μg lipid 781-1 + 300 μg rabies G vector

Group 3: 150 μg lipid 781-1 + 300 μg rabies G vector

Group 4: 75 μg lipid 781-1 + 300 μg rabies G vector

Group 5: 600 μg lipid 106-3 + 300 μg rabies G vector

Group 6: 300 μg lipid 106-3 + 300 μg rabies G vector

Group 7: 150 μg lipid 106-3 + 300 μg rabies G vector

Group 8: 300 μg lipid 4518-52 + 300 μg rabies G vector

Group 9: 300 μg lipid 106-3 + 300 μg rabies G vector (lyophilized and rehydrated)

Group 10: 75 μg lipid 106-3 + 75 μg rabies G vector.

Group 1 served as a control group to demonstrate immunogenicity of the naked DNA vaccine. Groups 2-4 were designed to determine if differences in the lipid-to-DNA ratio were important for lipid 781-1. Similarly, groups 5-7 were designed to determine if differences in the lipid-to-DNA ratio were important for lipid 106-3.

Group 8 was included to examine the efficacy of LTI lipid 4518-52. Group 9 was included to determine if lyophilization and rehydration of lipid:DNA complexes would improve cationic lipid vaccine efficacy in cats as previously demonstrated in mice by Gregoriadis, *ibid.* Finally, group 10 was included to determine is less than 300 μg of DNA could be used without affecting the ability of lipid 106-3 to enhance the ability of cats to elicit an immune response.

Rabies virus-specific neutralizing antibody activity was measured in the sera of all cats before and after the booster administration by RFFIT. Sera dilutions tested ranged from 1:5 to 1:174,693. Negative responses are listed as a titer of <1:5 while responses that are stronger than the final dilution tested are indicated by the ">" sign. Injections were made intramuscularly. It is known to those skilled in the art that an anti-rabies G antibody titer of 1:5 or greater, as measured by RFFIT, is protective. Results from these studies are shown in Table 2.

Table 2. Rabies G antibody titers of sera samples collected from cats administered a naked DNA vaccine or a complex of the present invention encoding rabies G.

Group 1

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHR5	Naked DNA (300 μg rabies G)	<1:5	1:25
BWM3	Naked DNA (300 μg rabies G)	<1:5	<1:5
3042	Naked DNA (300 μg rabies G)	<1:5	1:1400
ABO2	Naked DNA (300 μg rabies G)	<1:5	<1:5

Group 2

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHH1	300 μg DNA + 300 μg lipid 781-1	1:7000	1:167,449
3102	300 μg DNA + 300 μg lipid 781-1	1:1800	1:167,449
S72	300 μg DNA + 300 μg lipid 781-1	<1:5	1:50
QJB1	300 μg DNA + 300 μg lipid 781-1	<1:5	1:230

Group 3

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHN4	300 μg DNA + 150 μg lipid 781-1	<1:5	1:1400
QIN5	300 μg DNA + 150 μg lipid 781-1	1:2200	1:42,724
QGN5	300 μg DNA + 150 μg lipid 781-1	1:625	1:113,264
QHG1	300 μg DNA + 150 μg lipid 781-1	<1:5	1:7000

Group 4

cat #	Formulation	Titer Pre-boost	Titer Post-boost
QHR1	300 μg DNA + 75 μg lipid 781-1	<1:5	1:50
QIN2	300 μg DNA + 75 μg lipid 781-1	1:280	1:5100
QGR5	300 μg DNA + 75 μg lipid 781-1	1:2400	1:6000
ACN1	300 μg DNA + 75 μg lipid 781-1	<1:5	1:125

Group 5

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3603	300 μg DNA + 600 μg lipid 106-3	1:7000	1:174,693
BNJ2	300 μg DNA + 600 μg lipid 106-3	<1:5	1:1800
ZAH1	300 μg DNA + 600 μg lipid 106-3	1:5100	1:159,751
S203	300 μg DNA + 600 μg lipid 106-3	1:6300	1:67,491

Group 6

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3525	300 μg DNA + 300 μg lipid 106-3	1:280	1:45,668
BNJ1	300 μg DNA + 300 μg lipid 106-3	1:125	1:6800
BMX2	300 μg DNA + 300 μg lipid 106-3	<1:5	1:6800
S197	300 μg DNA + 300 μg lipid 106-3	>1:167,449	1:6800

Group 7

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3553	300 μg DNA + 150 μg lipid 106-3	1:1100	1:53,888
BN13	300 μg DNA + 150 μg lipid 106-3	<1:5	1:3125
E490	300 μg DNA + 150 μg lipid 106-3	1:25	1:6800
S192	300 μg DNA + 150 μg lipid 106-3	1:40	1:6000

Group 8

cat #	Formulation	Titer Pre-boost	Titer Post-boost
3541	300 μg DNA+300 μg lipid 4518-52	1:45	1:7000
BNH4	300 μg DNA+300 μg lipid 4518-52	1:1200	1;142,858
BLR1	300 μg DNA+300 μg lipid 4518-52	1:280	1:7000
S189	300 μg DNA+300 μg lipid 4518-52	>1:7000	1:159,751

Group 9

cat #	Formulation	Titer Pre-boost	Titer Post-boost
	DNA + lipid 106-3, dehyd &.rehyd[1]	1:2700	1:142,858
BNF4	DNA + lipid 106-3, dehyd &.rehyd[1]	1:170	>1:167,449
E457	DNA + lipid 106-3, dehyd &.rehyd[1]	1:1400	1:8,125
S186	DNA + lipid 106-3, dehyd &.rehyd[1]	1:45	1:3,125

[1]300 μg rabies G DNA + 300 μg lipid 106-3/dehydrated and rehydrated by the method of Gregoriadis, et al., *ibid.*

Group 10

cat #	Formulation	Titer Pre-boost	Titer Post-boost
BMC1	DNA, 75 μg + lipid 106-3, 75 μg	1:1800	1:6000
E451	DNA, 75 μg + lipid 106-3, 75 μg	1:3125	1:38,206
QNV1	DNA, 75 μg + lipid 106-3, 75 μg	1:360	1:34,600
ZAF1	DNA, 75 μg + lipid 106-3, 75 μg	1:440	1:5,400

Geometric Mean Titers for each group, pre-boost and post boost, for each group

Group	Formulation	mean titer, pre-	mean titer, post-
1	Naked DNA (300 μg)	<5	30.6
2	DNA, 300 μg + lipid 781-1, 300 μg (1:1)	133	4238
3	DNA, 300 μg + lipid 781-1, 150 μg (1:0.5)	77	14,756
4	DNA, 300 μg + lipid 781-1, 75 μg (1:0.25)	64	661
5	DNA, 300 μg + lipid 106-3, 600 μg (1:2)	1029	42,910
6	DNA, 300 μg + lipid 106-3, 300 μg (1:1)	413	10,409
7	DNA, 300 μg + lipid, 106-3, 150 μg (1:0.5)	48	9104
8	DNA, 300 μg + lipid 4518-52, 300 μg (1:1)	570	32,518
9	DNA, 300 μg + lipid, 106-3, 300 μg dehyd& rehyd[1]	412	49,159
10	DNA, 75 μg + lipid 106-3, 75 μg (1:1)	972	14,385

The data presented in Table 2 support the following conclusions: (1) in the cats receiving the naked DNA vaccine, no seroconversion was observed following the primary administration of vaccine immunization. In contrast, all of the cats receiving a nucleic acid molecule - cationic lipid complex of the present invention exhibited seroconversion after the booster administration, and at least 50% of the cats seroconverted per group after the initial administration of the complex. The best seroconversion was seen in groups 8, 9, and 10 in which 100% seroconversion was observed following the primary administration of complex. These results (0% seroconversion in group 1 and 100% seroconversion in groups 8-10 following the primary administration) were statistically significant by Fisher's exact test ($P<0.05$). (2) Following the booster administration, all nine groups that were administered a complex of the present invention exhibited stronger responses than the naked DNA vaccine control group. Despite the small number of cats in each group, statistically significant enhancement by Student's t test was observed in groups 5 and 9 as compared to group 1, i.e. naked DNA vaccine. (3) Varying the ratio of lipid-to-DNA did not have significant impact on the degree of enhancement (groups 2-4 and 5-7). (4) Dehydration and rehydration of the lipid:DNA complexes (lipid 106-3) prior to inoculation resulted

in 100% seroconversion following the primary administration and very strong responses in all cats following the boost (group 9). (5) Reducing the DNA dosage to 75 μg from 300 μg did not result in any loss of the enhancement potential since 100% seroconversion was observed after the primary administration of the complex and very strong responses were observed in all cats post-boost (group 10).

Example 7. Measuring luciferase expression in cat muscle.

Muscle and lymph node tissues were dissected and removed from the thigh of a sacrificed cat, see Example 3. The tissues were quick frozen on dry ice, and ground to a powder in liquid nitrogen. Ground frozen tissue was resuspended in 1X cell culture lysate reagent (25 mM Tris-Phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2 diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). After lysis, the cell debris was removed by centrifugation and supernatant was used in the following assay. An aliquot of the supernatant was mixed with Luciferase Assay Reagent, (Promega, Madison, WI). The mixture was placed in a Turner Designs Luminometer TD-20/20, (Promega), and the light emitted was measured for 15 seconds. The standard used to calibrate the assay was the recombinant firefly luciferase QUANTILUM™, (Promega).

Example 8. Comparison of expression of a DNA plasmid, formulated with and without LTI lipid 106-3, in the cat muscle.

In this example, evidence for increased antigen expression in the muscle upon formulation with lipid 106-3 was observed in an experiment in which 300 µg of a plasmid vector encoding luciferase was injected into each semimembranosus muscle (inner thigh) of a cat, one muscle receiving DNA complexed with lipid, and one muscle receiving naked DNA. In the case of DNA formulated with lipid 106-3, 300 µg of DNA was formulated with 300 µg of lipid 106-3. Specifically, the right thigh of the cat was injected with DNA alone; the left thigh was injected with DNA formulated with lipid 106-3. After 48 hours, the cat was sacrificed, the muscles were dissected and luciferase activity was measured as described in Example 7. Table 3 shows the luciferase assay standard curve used for this experiment, and Table 4 shows luminometer measurements for each dissected tissue in the cat.

Table 3. Luciferase assay standard curve

Sample	Luminometer readings
Blank	0.069
Positive control	332
2.5 µg standard	387.9
250 nanogram(ng) standard	54.07
25 ng standard	9.817
2.5 ng standard	1.909
250 picogram (pg) standard	0.431
25 pg standard	0.189

Table 4. Luminometer readings for each dissected muscle

muscle tested	Amount of tissue used in luciferase assay	Luminometer reading
Right superficial muscle (M. gracilis)	140 milligram (mg)	0.044
Right deep muscle (M. semimembranosus)	140 mg	0.065
Right Inguinal lymph node	100 mg	0.040
Right Popliteal lymph node	73 mg	0.052
Left superficial muscle	140 mg	0.058
Left Deep muscle	140 mg	13.82
Left Inguinal lymph node	could not locate	Not determined
Left Popliteal lymph node	80 mg	0.078

While no significant luciferase activity was observed in the leg injected with naked DNA, approximately 2 µg total of luciferase was produced in the entire deep muscle of the leg injected with the lipid/DNA formulation (assay sensitivity=2 pg), providing evidence for enhanced gene delivery and antigen production via use of cationic lipid formulations of the present invention.

Example 9. Effect of cationic lipid formulated DNA vaccines in mice.

This example demonstrates that formulation of DNA vaccines with cationic lipids does not enhance nucleic acid efficacy in mice, in contrast to the enhancement of nucleic acid efficacy seen in cats treated with cationic lipid/DNA formulations.

Three different nucleic acid molecules, encoding rabies glycoprotein G, were prepared as described in Example 2. The first, pMV 5044, contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the rabbit beta globin polyadenylation sequence. The second, pMV 5045, contains the CMV intron A promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence. The third, pMV 5046, contains the CMV promoter, the rabies glycoprotein G coding sequence, and the bovine growth hormone polyadenylation sequence.

The three nucleic acid molecules encoding rabies glycoprotein G (rabies G) were complexed with LTI lipid 106-3 at a lipid to DNA ratio (μg:μg) of 1:1 as described in Example 2. The corresponding "naked" DNA vaccines were prepared by dissolving the plasmids in saline.

A total of 30 mice were divided into six vaccine groups as follows:

Group 1 (pMV 5044, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 2 (pMV 5044, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 3 (pMV 5045, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 4 (pMV 5045, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 5 (pMV 5046, 50 μg + lipid): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Group 6 (pMV 5046, 100 μg alone): One injection, intramuscular. Antibody titers determined at four weeks post injection.

Table 5. Anti-rabies G antibody titers of sera samples collected from mice administered a naked DNA vaccine or a complex of the present invention encoding rabies G.

Group 1

mouse #	Formulation	Titer
1	pMV5044, 50 μg + Lipid 106-3	1:40
2	pMV5044, 50 μg + Lipid 106-3	1:40
3	pMV5044, 50 μg + Lipid 106-3	1:51
4	pMV5044, 50 μg + Lipid 106-3	1:115
5	pMV5044, 50 μg + Lipid 106-3	1:135

Group 2

mouse #	Formulation	Titer
1	pMV5044, 100 μg alone	1:43
2	pMV5044, 100 μg alone	1:53
3	pMV5044, 100 μg alone	1:242
4	pMV5044, 100 μg alone	1:1060
5	pMV5044, 100 μg alone	1:3795

Group 3

mouse #	Formulation	Titer
1	pMV5045, 50 μg + Lipid 106-3	1:40
2	pMV5045, 50 μg + Lipid 106-3	1:65
3	pMV5045, 50 μg + Lipid 106-3	1:68
4	pMV5045, 50 μg + Lipid 106-3	1:73
5	pMV5045, 50 μg + Lipid 106-3	1:137

Group 4

mouse #	Formulation	Titer
1	pMV5045, 100 μg alone	1:1
2	pMV5045, 100 μg alone	1:46
3	pMV5045, 100 μg alone	1:100
4	pMV5045, 100 μg alone	1:547
5	pMV5045, 100 μg alone	1:640

Group 5

mouse #	Formulation	Titer
1	pMV5046, 50 μg + Lipid 106-3	1:1
2	pMV5046, 50 μg + Lipid 106-3	1:1
3	pMV5046, 50 μg + Lipid 106-3	1:1
4	pMV5046, 50 μg + Lipid 106-3	1:34
5	pMV5046, 50 μg + Lipid 106-3	1:54

Group 6

mouse #	Formulation	Titer
1	pMV5046, 100 μg alone	1:1
2	pMV5046, 100 μg alone	1:1
3	pMV5046, 100 μg alone	1:1
4	pMV5046, 100 μg alone	1:1
5	pMV5046, 100 μg alone	1:59

Rabies-virus specific neutralizing antibody activity was measured by RFFIT in the sera of all mice four weeks after injection with three different nucleic acid molecules containing Rabies glycoprotein G.

The data presented in Table 5 indicate that cationic lipid formulation of a DNA vaccine does not enhance vaccine efficacy, as measured by humoral (antibody) response, in mice. These data are in contrast to results obtained in cats, where vaccine efficacy is enhanced by formulation with cationic lipids. For the nucleic acid construct pMV5044,

formulation with lipid actually appears to slightly reduce DNA vaccine efficacy for mice, with the geometric means (of the five mice per group) declining from 294 with DNA alone to 66 with DNA/lipid complex. Results from the other two constructs in mice also showed no increase in efficacy; the geometric means were as follows: for pMV5045, 69.4 for DNA alone and 70.7 with DNA/lipid complex; and for pMV5046, 2.3 for DNA alone and 4.5 for DNA/lipid complex.

Example 10. Administration of a DNA plasmid, formulated with and without LTI lipid 106-3, to cats.

This example demonstrates the local immune response at the site of injection of DNA plasmids formulated with or without LTI lipid 106-3. Each of four cats was administered each of the following formulations to each of the following sites on the ventral side: (a) saline (i.e., vehicle alone) to the right arm; (b) 300 μg of lipid 106-3 (lipid alone) to the left arm; (c) 300 μg of a naked plasmid vector encoding rabies glycoprotein G (naked rabies G vector) to the upper right foot; (d) 300 μg of a naked plasmid vector encoding luciferase (naked luciferase vector) to the lower right foot; (e) 300 μg of rabies G vector formulated with 300 μg of lipid 106-3 to the upper left foot; and (f) 300 μg of luciferase vector formulated with 300 μg of lipid 106-3 to the lower left foot.

Six days after administration of the various formulations, the cats were euthanized and muscle and popliteal lymph node muscles were collected. Although the injection sites were marked, it was difficult to obtain muscle samples from the injection sites; thus, only four injection sites were identified, namely those for the saline only and naked rabies G vector in one cat and those for lipid only and rabies G vector plus lipid in another cat. Muscle samples were sectioned using a cryostat and the sections were stained using hematoxylin and eosin to analyze the population of cells infiltrating the sites of injection. Muscle samples were also stained with antibodies specific for B-cells (anti-CD79a antibodies) using techniques known to those skilled in the art.

No differences were seen among the various lymph nodes with respect to cell infiltration. In the muscle samples where vehicle alone, lipid alone or naked rabies G vector was injected, the infiltrating population of cells were mostly macrophage-like cells. In contrast, in the muscle sample where the formulation comprising rabies

G vector complexed with lipid was infected, the infiltrating cells were predominantly lymphocyte-like cells. Staining results with anti-CD79a antibodies suggested that the majority of lymphocytes present were T cells.

These results, as well as others provided herein, suggest that administration of nucleic acid molecules complexed with cationic lipids to cats leads to enhanced expression of the protein encoded by the nucleic acid molecule and infiltration of lymphocytes to the injection site which apparently does not occur when naked nucleic acid molecules are administered in a similar manner. Without being bound by theory, it is believed that this infiltration of lymphocytes might explain the enhanced immune response seen with nucleic acid molecule-cationic lipid complexes of the present invention.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A method to elicit an immune response to an antigen in a felid, said method comprising parenterally administering to said felid a composition comprising a nucleic acid molecule complexed with a cationic lipid, wherein said nucleic acid molecule encodes said antigen.

2. A method to deliver a nucleic acid molecule to a felid, said method comprising parenterally administering a composition comprising said nucleic acid molecule complexed with a cationic lipid.

3. A method to protect a felid from rabies infection, said method comprising parenterally administering to said felid a composition comprising a nucleic acid molecule encoding rabies glycoprotein G, wherein said nucleic acid molecule is complexed with a cationic lipid.

4. The method of Claim 2, wherein said nucleic acid molecule encodes a compound selected from the group consisting of an RNA molecule and a protein.

5. The method of Claim 2, wherein said nucleic acid molecule encodes a protein that elicits an immune response in said felid.

6. The method of Claim 5, wherein said protein is selected from the group consisting of an antigen and an immunomodulator.

7. The method of Claim 1 or 5, wherein said immune response comprises an antibody response.

8. The method of Claim 1 or 5, wherein said immune response comprises a cell-mediated response.

9. The method of Claim 1 or 5, wherein said immune response protects said felid from disease.

10. The method of Claim 1 or 6, wherein said antigen is selected from the group consisting of a protozoan parasite antigen, a helminth parasite antigen, an ectoparasite antigen, a fungal antigen, a bacterial antigen, and a viral antigen.

11. The method of Claim 1 or 6, wherein said antigen is selected from the group consisting of a calicivirus antigen, a coronavirus antigen, a herpesvirus antigen, an immunodeficiency virus antigen, an infectious peritonitis virus antigen, a leukemia virus antigen, a parvovirus antigen, a rabies virus antigen, a *Bartonella* antigen, a *Yersinia*

antigen, a *Dirofilaria* antigen, a *Toxoplasma* antigen, a flea antigen, a flea allergen, a midge antigen, a midge allergen, a mite antigen, a mite allergen, and a tumor antigen.

12. The method of Claim 1 or 6, wherein said antigen comprises rabies glycoprotein G antigen.

13. The method of Claim 1, 2, or 3, wherein said cationic lipid comprises a tetramethyltetraalkyl spermine analog lipid.

14. The method of Claim 1 or 3, wherein said composition further encodes an immunomodulator.

15. The method of Claim 1, 2, or 3, wherein said felid is selected from the group consisting of domestic cats, wild cats, and zoo cats.

16. The method of Claim 1, 2, or 3, wherein the felid is selected from the group consisting of domestic cats, lions, tigers, leopards, panthers, cougars, bobcats, lynx, bobcats, lynx, jaguars, cheetahs, and servals.

17. The method of Claim 1, 2, or 3, wherein the felid is a domestic cat.

18. The method of Claim 1, 3, or 5, wherein a single administration of said composition elicits an immune response.

19. The method of Claim 1, 3, or 6, wherein said step of administering enhances an immune response compared to administration of a naked DNA vaccine encoding said antigen of Claim 1 or 6 or said rabies glycoprotein G of Claim 3 to a felid.

20. The method of Claim 1, 2, or 3, wherein said step of administering is selected from the group of intramuscular administration, intravenous administration, subcutaneous administration, intradermal administration, and intraperitoneal administration.

21. The method of Claim 1, 2, or 3, wherein said step of administering effects about 75% seroconversion in a population of felids administered said nucleic acid molecule.

22. The method of Claim 1, 2, or 3, wherein said step of administering effects about 100% seroconversion in a population of felids administered said nucleic acid molecule.

23. The method of Claim 1, 2, or 3, wherein said nucleic acid molecule:lipid ratio is from about 1:10 to about 10:1.

24. The method of Claim 1, 2, or 3, wherein said nucleic acid molecule is administered in a dose of from about 75 micrograms to about 1,000 micrograms.

25. The method of Claim 1, 2 or 3, wherein said nucleic acid molecule is administered in a dose of not more than about 75 micrograms.

26. The method of Claim 1, 2, or 3, wherein said composition is dehydrated and subsequently rehydrated prior to administration.

27. The method of Claim 1, 2, or 3, wherein said composition further comprises an excipient.

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

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To:

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Date of mailing (day/month/year) 29 June 2000 (29.06.00)	
International application No. PCT/US99/24769	**Applicant's or agent's file reference** HKZ-034CPPC
International filing date (day/month/year) 22 October 1999 (22.10.99)	**Priority date** (day/month/year) 23 October 1998 (23.10.98)
Applicant HAYNES, Joel, R. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

23 May 2000 (23.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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To:

HANLEY, Elizabeth, A.
Lahive & Cockfield, LLP
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NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	13.12.2000
Applicant's or agent's file reference: HKZ-034CPPC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/24769	22/10/1999	23/10/1998

Applicant: HESKA CORPORATION et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
HKZ-034CPPC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/24769	22/10/1999	23/10/1998
International Patent Classification (IPC) or national classification and IPC C12N15/47		
Applicant HESKA CORPORATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/05/2000	13.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Valcarcel, R Telephone No. +49 89 2399 2368

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/24769

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-33 as originally filed

Claims, No.:

1-27 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	NONE
Inventive step (IS)	Yes:	Claims	NONE
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	No opinion given
	No:	Claims	No opinion given

2. Citations and explanations
   s s parat sh t

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents; the numbering corresponds to the order of citation in the International Search Report:

D1: WO 95 30019 A (THE UPJOHN COMPANY) 9 November 1995 (1995-11-09)

D3: NORMAN J ET AL: 'Development of improved vectors for DNA-based immunization and other gene therapy applications.' VACCINE, vol. 15, no. 8, June 1997 (1997-06), pages 801-3

The documents D6 and D7 were not cited in the international search report. D7 has been cited by the applicant in the description.

D6: XIANG, ZHI QUAN ET AL: 'A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier', VIROLOGY, , 1996, vol. 219 , pages 220 to 227

D7: MCCLUSKIE, MJ ET AL.: 'Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA', ANTISENSE NUCLEIC ACID DRUG DEV, October 1998, vol. 8, pages 401 to 414

The exact publication date of D7 is not available yet, but it has been inquired to the Journal's editorial office.

Cited on (408')

Re Item IV

Since complexes of cationic lipids with nucleic aci :d in animals in the prior art in order to elicit immune responses to ... :y the nucleic acid molecule (see for example D3), there is **no special technical feature** (in the sense of Rule 13.2) which links the methods referred to in claims 1 to 27. **Each possible antigen used in a method to elicit an immune response in a felid would constitute a separate invention.** Examples are methods wherein the antigen is one selected from the list of antigens recited in the claims. Thus, there is a vast amount of different inventions which are not unitarily linked, and therefore, **the present application lacks unity.** However, the IPEA elected to carry out examination on the subject-matter of all claims.

R Item V

1. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claims 1 and 2 does not involve an inventive step**.

D1 (which is considered to represent the closest prior art) discloses a method to elicit an immune response to an antigen in a felid (cat) and a method to deliver a nucleic acid molecule to a felid, said methods comprising parenterally administering to said felid a composition comprising a nucleic acid molecule wherein said nucleic acid molecule encodes said antigen.

The subject-matter of claims 1 and 2 differ from the methods disclosed in D1 in that in the methods of claims 1 and 2 cationic lipids are complexed with the nucleic acid molecule.

The technical problem would have been to provide alternative (improved) methods to elicit an immune response to an antigen in a felid (claim 1) or to deliver a nucleic acid molecule to a felid (claim 2). The solution of the present application is to complex the nucleic acid molecule with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

D3 discloses that cationic lipids had been known to enhance the delivery and expression of plasmid DNA *in vitro* and *in vivo*. In particular, D3 discloses that the particular cationic lipid GAP-DLRIE greatly enhances the expression of plasmid DNA delivered to mouse lung (see page 802, right column, second paragraph). D3 discloses that GAP-DLRIE may be a useful lipid for the administration of plasmid DNA to elicit an immune response in animals (see lines bridging left and right columns of page 803).

D7 (cited by the applicant on page 11 of the description, lines 1 to 3) discloses that cationic lipids enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). As cited in the description of the present application (see paragraph linking pages 2 and 3) there were at least two other documents in the prior art which disclosed that cationic lipids formulated together with DNA vaccines enhanced the immune response as compared to the "naked" DNA.

In order to solve the underlying technical problem, the person skilled in the art (in view of the technical teachings of D3, D7, or the documents cited in the description, see above), would have been motivated to complex cationic lipids with the DNA molecules used in the methods of D1 and would have reached the solutions according to claims 1 and 2. Therefore, the subject-matter of claims 1 and 2 does not involve an inventive step.

2. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 3 does not involve an inventive step**.

D6 (considered as the closest prior art) discloses a method to protect animals from rabies infection, said method comprising administering parenterally to said animals a composition comprising a nucleic acid molecule encoding **rabies glycoprotein G** (see abstract).

The technical problem would have been to provide an alternative (improved) method to protect animals (*inter alia* felids) from rabies infection. The solution of the present application is to complex the nucleic acid molecule encoding rabies glycoprotein G with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

As discussed on point 1 of the present communication (see above), several prior art documents (as D3, D7, or the two documents cited in the present application in the paragraph bridging pages 2 and 3 of the description) disclose the use of cationic lipids to enhance the uptake and expression of plasmid DNA and consequently the immune response elicited by the antigen encoded by said DNA.

Thus, a person skilled in the art would have been motivated to complex the DNA encoding rabies glycoprotein G with cationic lipids in order to enhance the immune response elicited by the DNA molecule encoding rabies glycoprotein G and therefore, to enhance the protection against rabies infection. Therefore, the subject-matter of claim 3 does not involve an inventive step.

3. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 13 does not involve an inventive step.**

   D7 (cited by the applicants on page 11, lines 1 to 3, of the description) discloses that certain cationic lipids (tetramethyltetraalkylspermine analogs) enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). These cationic lipids were obvious candidates to be used in the methods of the present application, and therefore, the subject-matter of claim 13 does not involve an inventive step.

4. **Claims 4 to 12 and 14 to 27 do not contain any features** which, in combination with the features of any claim to which they refer, **meet the requirements of the PCT in respect of inventive step.**

5. Claims 1 to 27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

   For the assessment of the present claims 1 to 27 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to methods for treatment of the human or animal body by surgery or therapy, or to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO99/66879	22 December 1999	22 June 1999	25 June 1998

Re Item VIII

Claim 3 does not meet the requirements of Article 5 PCT. It has not been shown in the application as filed **whether the felids** to which the composition (comprising a nucleic acid molecule encoding rabies glycoprotein G complexed with a cation lipid) was administered, **are indeed protected from rabies infection.**

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
HKZ-034CPPC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/24769	22/10/1999	23/10/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/47		
Applicant		
HESKA CORPORATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/05/2000	13.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Valcarcel, R Telephone No. +49 89 2399 2368

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/24769

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-33 as originally filed

Claims, No.:

1-27 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	NONE
Inventive step (IS)	Yes:	Claims	NONE
	No:	Claims	1-27
Industrial applicability (IA)	Yes:	Claims	No opinion given
	No:	Claims	No opinion given

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents; the numbering corresponds to the order of citation in the International Search Report:

D1: WO 95 30019 A (THE UPJOHN COMPANY) 9 November 1995 (1995-11-09)

D3: NORMAN J ET AL: 'Development of improved vectors for DNA-based immunization and other gene therapy applications.' VACCINE, vol. 15, no. 8, June 1997 (1997-06), pages 801-3

The documents D6 and D7 were not cited in the international search report. D7 has been cited by the applicant in the description.

D6: XIANG, ZHI QUAN ET AL: 'A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier', VIROLOGY, , 1996, vol. 219 , pages 220 to 227

D7: MCCLUSKIE, MJ ET AL.: 'Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA', ANTISENSE NUCLEIC ACID DRUG DEV, October 1998, vol. 8, pages 401 to 414

The exact publication date of D7 is not available yet, but it has been inquired to the Journal's editorial office.

Re Item IV

Since complexes of cationic lipids with nucleic acid molecules have been used in animals in the prior art in order to elicit immune responses to the antigen encoded by the nucleic acid molecule (see for example D3), there is **no special technical feature** (in the sense of Rule 13.2) which links the methods referred to in claims 1 to 27. **Each possible antig n used in a method to elicit an immune response in a felid would constitute a separat invention.** Examples are methods wherein the antigen is one selected from the list of antigens recited in the claims. Thus, there is a vast amount of different inventions which are not unitarily linked, and therefore, **th present application lacks unity.** However, the IPEA elected to carry out examination on the subject-matter of all claims.

Re Item V

1. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claims 1 and 2 does not involve an inventive step.**

D1 (which is considered to represent the closest prior art) discloses a method to elicit an immune response to an antigen in a felid (cat) and a method to deliver a nucleic acid molecule to a felid, said methods comprising parenterally administering to said felid a composition comprising a nucleic acid molecule wherein said nucleic acid molecule encodes said antigen.

The subject-matter of claims 1 and 2 differ from the methods disclosed in D1 in that in the methods of claims 1 and 2 cationic lipids are complexed with the nucleic acid molecule.

The technical problem would have been to provide alternative (improved) methods to elicit an immune response to an antigen in a felid (claim 1) or to deliver a nucleic acid molecule to a felid (claim 2). The solution of the present application is to complex the nucleic acid molecule with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

D3 discloses that cationic lipids had been known to enhance the delivery and expression of plasmid DNA *in vitro* and *in vivo*. In particular, D3 discloses that the particular cationic lipid GAP-DLRIE greatly enhances the expression of plasmid DNA delivered to mouse lung (see page 802, right column, second paragraph). D3 discloses that GAP-DLRIE may be a useful lipid for the administration of plasmid DNA to elicit an immune response in animals (see lines bridging left and right columns of page 803).

D7 (cited by the applicant on page 11 of the description, lines 1 to 3) discloses that cationic lipids enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). As cited in the description of the present application (see paragraph linking pages 2 and 3) there were at least two other documents in the prior art which disclosed that cationic lipids formulated together with DNA vaccines enhanced the immune response as compared to the "naked" DNA.

In order to solve the underlying technical problem, the person skilled in the art (in view of the technical teachings of D3, D7, or the documents cited in the description, see above), would have been motivated to complex cationic lipids with the DNA molecules used in the methods of D1 and would have reached the solutions according to claims 1 and 2. Therefore, the subject-matter of claims 1 and 2 does not involve an inventive step.

2. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 3 does not involve an inventive step**.

D6 (considered as the closest prior art) discloses a method to protect animals from rabies infection, said method comprising administering parenterally to said animals a composition comprising a nucleic acid molecule encoding **rabies glycoprotein G** (see abstract).

The technical problem would have been to provide an alternative (improved) method to protect animals (*inter alia* felids) from rabies infection. The solution of the present application is to complex the nucleic acid molecule encoding rabies glycoprotein G with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

As discussed on point 1 of the present communication (see above), several prior art documents (as D3, D7, or the two documents cited in the present application in the paragraph bridging pages 2 and 3 of the description) disclose the use of cationic lipids to enhance the uptake and expression of plasmid DNA and consequently the immune response elicited by the antigen encoded by said DNA.

Thus, a person skilled in the art would have been motivated to complex the DNA encoding rabies glycoprotein G with cationic lipids in order to enhance the immune response elicited by the DNA molecule encoding rabies glycoprotein G and therefore, to enhance the protection against rabies infection. Therefore, the subject-matter of claim 3 does not involve an inventive step.

3. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 13 does not involve an inventive step.**

D7 (cited by the applicants on page 11, lines 1 to 3, of the description) discloses that certain cationic lipids (tetramethyltetraalkylspermine analogs) enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). These cationic lipids were obvious candidates to be used in the methods of the present application, and therefore, the subject-matter of claim 13 does not involve an inventive step.

4. **Claims 4 to 12 and 14 to 27 do not contain any features** which, in combination with the features of any claim to which they refer, **meet the requirements of th PCT in respect of inventive step.**

5. Claims 1 to 27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present claims 1 to 27 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to methods for treatment of the human or animal body by surgery or therapy, or to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO99/66879	22 December 1999	22 June 1999	25 June 1998

Re Item VIII

Claim 3 does not meet the requirements of Article 5 PCT. It has not been shown in the application as filed **whether the felids** to which the composition (comprising a nucleic acid molecule encoding rabies glycoprotein G complexed with a cation lipid) was administered, **are indeed protected from rabies infection.**

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
HANLEY, Elizabeth, A.
Lahive & Cockfield, LLP
28 State Street
Boston, MA 02109
ETATS-UNIS D'AMERIQUE

DOCKETED
Oct. 12, 2000 - 5 day letter
Oct. 17, 2000 - Written opinion

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing (day/month/year)	17.08.2000
REPLY DUE	**within 2 month(s) and 15 days** from the above date of mailing
Applicant's or agent's file reference	HKZ-034CPPC
International application No.	PCT/US99/24769
International filing date (day/month/year)	22/10/1999
Priority date (day/month/year)	23/10/1998
International Patent Classification (IPC) or both national classification and IPC	C12N15/47
Applicant	HESKA CORPORATION et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain document cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 23/02/2001.

RECEIVED
LAHIVE & COCKFIELD
DOCKET DEPT.
AUG 23 2000

RETRIEVED 8/25/00
FORWARDED

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Valcarcel, R

Formalities officer (incl. extension of time limits)
Vullo, G
Telephone No. +49 89 2399 8061

Form PCT/IPEA/408 (cover sheet) (January 1994)

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-33 as originally filed

Claims, No.:

1-27 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees, the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees:

se separat sh t

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	
Inventive step (IS)	Claims	1-27 (NO)
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Reference is made to the following documents; the numbering corresponds to the order of citation in the International Search Report:

D1: WO 95 30019 A (THE UPJOHN COMPANY) 9 November 1995 (1995-11-09)

D3: NORMAN J ET AL: 'Development of improved vectors for DNA-based immunization and other gene therapy applications.' VACCINE, vol. 15, no. 8, June 1997 (1997-06), pages 801-3

The documents D6 and D7 were not cited in the international search report. Copies of D6 and D7 are appended hereto. D7 has been cited by the applicant in the description.

D6: XIANG, ZHI QUAN ET AL: 'A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier', VIROLOGY, , 1996, vol. 219 , pages 220 to 227

D7: MCCLUSKIE, MJ ET AL.: 'Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA', ANTISENSE NUCLEIC ACID DRUG DEV, October 1998, vol. 8, pages 401 to 414

The exact publication date of D7 is not available yet, but it has been inquired to the Journal's editorial office.

Re Item IV

Since complexes of cationic lipids with nucleic acid molecules have been used in animals in the prior art in order to elicit immune responses to the antigen encoded by the nucleic acid molecule (see for example D3), there is **no special technical feature** (in the sense of Rule 13.2) which links the methods referred to in claims 1 to 27. **Each possible antigen used in a method to elicit an immune response in a felid would constitute a separate invention.** Examples are methods wherein the antigen is one selected from the list of antigens recited in the claims. Thus, there is a vast amount of different inventions which are not unitarily linked, and therefore, **the present application lacks unity.** However, the IPEA elected to carry out examination on the subject-matter of all claims.

Re Item V

1. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claims 1 and 2 does not involve an inventive step**.

D1 (which is considered to represent the closest prior art) discloses a method to elicit an immune response to an antigen in a felid (cat) and a method to deliver a nucleic acid molecule to a felid, said methods comprising parenterally administering to said felid a composition comprising a nucleic acid molecule wherein said nucleic acid molecule encodes said antigen.

The subject-matter of claims 1 and 2 differ from the methods disclosed in D1 in that in the methods of claims 1 and 2 cationic lipids are complexed with the nucleic acid molecule.

The technical problem would have been to provide alternative (improved) methods to elicit an immune response to an antigen in a felid (claim 1) or to deliver a nucleic acid molecule to a felid (claim 2). The solution of the present application is to complex the nucleic acid molecule with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

D3 discloses that cationic lipids had been known to enhance the delivery and expression of plasmid DNA *in vitro* and *in vivo*. In particular, D3 discloses that the particular cationic lipid GAP-DLRIE greatly enhances the expression of plasmid DNA delivered to mouse lung (see page 802, right column, second paragraph). D3 discloses that GAP-DLRIE may be a useful lipid for the administration of plasmid DNA to elicit an immune response in animals (see lines bridging left and right columns of page 803).

D7 (cited by the applicant on page 11 of the description, lines 1 to 3) discloses that cationic lipids enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). As cited in the description of the present application (see paragraph linking pages 2 and 3) there were at least two other documents in the prior art which disclosed that cationic lipids formulated together with DNA vaccines enhanced the immune response as compared to the "naked" DNA.

In order to solve the underlying technical problems, the person skilled in the art (in view of the technical teachings of D3, D7, or the documents cited in the description, see above), would have been motivated to complex cationic lipids with the DNA molecules used in the methods of D1 and would have reached the solutions according to claims 1 and 2. Therefore, the subject-matter of claims 1 and 2 does not involve an inventive step.

2. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 3 does not involve an inventive step**.

D6 (considered as the closest prior art) discloses a method to protect animals from rabies infection, said method comprising administering parenterally to said animals a composition comprising a nucleic acid molecule encoding **rabies glycoprotein G** (see abstract).

The technical problem would have been to provide an alternative (improved) method to protect animals (*inter alia* felids) from rabies infection. The solution of the present application is to complex the nucleic acid molecule encoding rabies glycoprotein G with cationic lipids. The solution is not considered to involve an inventive step for the following reasons.

As discussed on point 1 of the present communication (see above), several prior art documents (as D3, D7, or the two documents cited in the present application in the paragraph bridging pages 2 and 3 of the description) disclose the use of cationic lipids to enhance the uptake and expression of plasmid DNA and consequently the immune response elicited by the antigen encoded by said DNA.

Thus, a person skilled in the art would have been motivated to complex the DNA encoding rabies glycoprotein G with cationic lipids in order to enhance the immune response elicited by the DNA molecule encoding rabies glycoprotein G and therefore, to enhance the protection against rabies infection. Therefore, the subject-matter of claim 3 does not involve an inventive step.

3. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of **claim 13 does not involve an inventive step.**

D7 (cited by the applicants on page 11, lines 1 to 3, of the description) discloses that certain cationic lipids (tetramethyltetraalkylspermine analogs) enhance the transfection efficiency of plasmid DNA into animal cells (also parenteral administration was used). These cationic lipids were obvious candidates to be used in the methods of the present application, and therefore, the subject-matter of claim 13 does not involve an inventive step.

4. **Claims 4 to 12 and 14 to 27 do not contain any features** which, in combination with the features of any claim to which they refer, **meet the requirements of th PCT in respect of inventive step.**

5. Claims 1 to 27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present claims 1 to 27 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to methods for treatment of the human or animal body by surgery or therapy, or to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

6. **It is not at present apparent which part of the application could serve as a basis for a claim which meets the criteria of the PCT. Should the applicant nevertheless regard some particular matter as acceptable under the PCT, an**

independent claim should be filed taking account of Article 34 (2)(b) PCT.

Re Item VI

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO99/66879	22 December 1999	22 June 1999	25 June 1998

Re Item VIII

Claim 3 does not meet the requirements of Article 5 PCT. It has not been shown in the application as filed **whether the felids** to which the composition (comprising a nucleic acid molecule encoding rabies glycoprotein G complexed with a cation lipid) was administered, **are indeed protected from rabies infection.**

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
HKZ-034CPPC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 24769	22/10/1999	23/10/1998
Applicant		
HESKA CORPORATION et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of th figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/24769

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/47 A61K48/00 A61P31/04 A61P31/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 30019 A (THE UPJOHN COMPANY) 9 November 1995 (1995-11-09) claims 1-27	1,2,4-27
Y	US 4 726 946 A (BASS ET AL) 23 February 1998 (1998-02-23) column 1, line 9 - line 17	3
Y	NORMAN J ET AL: "Development of improved vectors for DNA-based immunization and other gene therapy applications." VACCINE, vol. 15, no. 8, June 1997 (1997-06), pages 801-3, XP002135482 abstract	1-27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 April 2000	03/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/24769

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	OSORIO J ET AL: "Immunization of gogs and cats with a DNA vaccine against rabies virus." VACCINE, vol. 17, no. 9-10, 5 March 1999 (1999-03-05), pages 1109-16, XP002135483 the whole document	1-27
T	WO 99 66879 A (NATIONAL JEWISH MEDICAL AND RESEARCH CENTER) 29 December 1999 (1999-12-29) claims 1-115	1-27

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir. .national application No.
PCT/US 99/24769

B x I Observations where certain claims w re found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for th following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by th applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/US 99/24769

Patent document cited in search report	Publication date	Patent family member(s)	Publication dat
WO 9530019 A	09-11-1995	AU 2234995 A	29-11-1995
		EP 0758396 A	19-02-1997
		JP 9512177 T	09-12-1997
		US 5833993 A	10-11-1998
US 4726946 A	23-02-1988	US 4347239 A	31-08-1982
		AU 544338 B	23-05-1985
		AU 6714381 A	04-02-1982
		CA 1183452 A	05-03-1985
		CA 1198368 C	24-12-1985
		CA 1205380 C	03-06-1986
		EP 0044920 A	03-02-1982
		ES 500199 D	16-01-1982
		ES 8202357 A	16-04-1982
		HK 50184 A	29-06-1984
		IE 50889 B	06-08-1986
		JP 1855404 C	07-07-1994
		JP 5055487 B	17-08-1993
		JP 57032227 A	20-02-1982
		KR 8401514 B	29-09-1984
		NZ 196214 A	31-07-1984
		PH 17383 A	06-08-1984
		PH 18743 A	16-09-1985
		PH 22308 A	22-07-1988
		SG 3384 G	01-02-1985
		US 4429045 A	31-01-1984
		US 4584194 A	22-04-1986
		US 4711778 A	08-12-1987
		ZA 8101042 A	26-05-1982
WO 9966879 A	29-12-1999	NONE	

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

HANLEY, Elizabeth, A.
Lahive & Cockfield, LLP
28 State Street
Boston, MA 02109
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 17 December 1999 (17.12.99)	
Applicant's or agent's file reference HKZ-034CPPC	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/24769	International filing date (day/month/year) 22 October 1999 (22.10.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 23 October 1998 (23.10.98)
Applicant HESKA CORPORATION et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
23 Octo 1998 (23.10.98)	60/105,469	US	10 Dece 1999 (10.12.99)
02 Marc 1999 (02.03.99)	60/122,446	US	10 Dece 1999 (10.12.99)

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DEC 27 1999
Retrieved 1/7/00
Entered
BY

The International Bur au of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

C. Carrié

Telephone No. (41-22) 338.83.38

003020252

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

T :
HANLEY, Elizabeth, A.
Lahiv & Cockfield, LLP
28 State Street
Boston, MA 02109
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)	
04 May 2000 (04.05.00)	
Applicant's or agent's file reference HKZ-034CPPC	**IMPORTANT NOTICE**
International application No. PCT/US99/24769	**International filing date** (day/month/year) 22 October 1999 (22.10.99)
Priority date (day/month/year) 23 October 1998 (23.10.98)	
Applicant HESKA CORPORATION et al	

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,JP,KP,KR,MA,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no c py of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE, GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA, PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   04 May 2000 (04.05.00) under No. WO 00/24428

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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DOCKET DEPT.
MAY 15 2000
RETRIEVED: 6/12
FORWARDED: NFB 6/14
3248628

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/308 (July 1996)